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CHARACTERIZATION OF A CAUSATIVE AGENT
OF VIRUS PNEUMONIA OF PIGS (VPP).

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CHARACTERIZATION OF A CAUSATIVE AGENT
OF VIRUS PNEUMONIA OF PIGS (VPP)

by

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INTRODUCTION

Modern systems of swine management in which large numbers of pigs are confined in close contact create an ideal environment for the spread of respiratory diseases. The most widespread of these diseases, and the one considered by many to be the most important disease of swine in the world today, is the condition variously referred to as "Virus Pneumonia of Pigs," "Enzootic Pneumonia of Pigs," "Infectious Pneumonia" or "Enzootic Virus Pneumonia."

Virus pneumonia of pigs is a chronic pneumonia characterized by a persistent nonproductive cough, loss of condition, growth retardation and low mortality. The first signs of the disease are usually manifested 2 to 3 weeks after exposure. Gross pneumonic lesions occur predominantly in the anterior lobes and are characterized on microscopic examination by pronounced peribronchiolar and perivasculär lymphoid hyperplasia, thickening of the alveolar walls, and septal cell proliferation.

The etiology of virus pneumonia of pigs has not been elucidated, and there is at present no adequate means of comparing the chronic pneumonias of swine occurring in different parts of the world. However, several investigators have reported the isolation of agents from virus pneumonia of pigs, and some of these agents have been used to reproduce microscopic and on occasion macroscopic lesions of the disease.

The respiratory disease-free status of the experimental animals used in these experiments was in most cases not defined, and failure to recognize the possibility of intercurrent infections with known Mycoplasma spp. has further confused the situation, making it difficult to evaluate the etiological significance of the agents isolated.

Small pleomorphic organisms have been observed in touch preparations prepared from pneumonic lung, and in cell cultures infected with the agent of virus pneumonia of pigs. The relationship between these organisms and the causative agent of the disease has not been clearly defined, but it has been suggested that the organisms may be the cause of the disease.

In view of the confusing and often contradictory information in current literature on virus pneumonia of pigs and the lack of knowledge concerning the nature of the agent causing the disease in the United States, it was considered essential to characterize and define a causative agent of the disease, and if possible to develop methods for its propagation. This study was undertaken in an attempt to attain these objectives.

REVIEW OF THE LITERATURE

The literature review covers 4 areas: 1. virus pneumonia of pigs, 2. human primary atypical pneumonia, 3. ultrastructure of Mycoplasma, and 4. ultrastructure of psittacosis agents.

Virus Pneumonia of Pigs

Pneumonia has been recognized as an important disease problem in swine for over two thousand years. Aristotle (329 B.C.) in his "Historia Animalium" wrote, "the pig suffers from three diseases, one of which is called branchos", and then he described the termination of this disease as follows: "the decay goes on until it reaches the lungs, when the animal succumbs."

Very little progress was made in the elucidation of the etiology of swine pneumonias until Shope (1931a, 1931b) and Lewis and Shope (1931) demonstrated that Haemophilus influenza-suis and a virus were involved as causative agents of swine influenza. Their work represents a major milestone in the progress toward better understanding of the complex etiology of swine pneumonias.

Gulrajani and Beveridge (1951) recognized a chronic pneumonia of swine in Britain which differed from swine influenza, and from which they could not isolate swine influenza virus. They did however manage to reproduce the disease in

susceptible pigs with material filtered through Gradocal membranes of 0.8μ APD, but not with 0.56μ APD membrane filtrates. Macroscopic pneumonic lesions were present on the twelfth day postinoculation. They failed to propagate the infectious agent in the allantoic cavity or on the chorio-allantoic membrane of embryonated hen's eggs, and they could not infect mice or ferrets with the agent. They recognized that their disease resembled the chronic pneumonia described by Pullar (1948, 1949a, 1949b) in Australia, and called the disease by the name suggested by him, namely "Infectious Pneumonia of Pigs."

The emphasis in this review will be on the attempts at propagation and characterization of the causative agent of virus pneumonia of pigs since excellent reviews by Betts (1953), Hjärre (1957), L'Ecuyer et al. (1961), L'Ecuyer (1962) and Young (1964a, 1964b) adequately cover the historical, geographic and economic aspects of the disease.

L'Ecuyer (1962), in a very comprehensive review of the literature pertaining to pneumonia in swine, discussed the emergence of virus pneumonia of pigs as a recognizable entity, differing from swine influenza on clinical, pathological and etiological basis. He also presented a detailed discussion of the role of a number of bacterial species and Mycoplasma hyorhinis as primary or secondary pathogens in swine pneumonia.

Shope (1931a), while studying field outbreaks of swine influenza in eastern Iowa, commented on a form of the disease differing from the typical "hog flu". This condition was characterized by greater chronicity, the absence of prostration, and by sporadic occurrence in the affected herds. Lewis and Shope (1931), in commenting on a report by Spray, questioned the validity of his work as follows: "since Spray did not differentiate infectious pneumonia, hemorrhagic septicemia, and influenza, his findings are not of great value." It is clear that these workers recognized what they considered to be a separate infectious pneumonia of pigs.

Betts (1952) suggested the name "Virus Pneumonia of Pigs", (VPP) for the disease described by Gulrajani and Beveridge. The main criterion upon which the decision to use the term "virus" rested was the result of filtration experiments which indicated that the agent was "probably not less than 200 m μ in diameter". This name has become widely accepted, particularly in the United States.

The serial transmission of "infectious pneumonia" in susceptible pigs described by Gulrajani and Beveridge (1951) was successfully repeated by Hjärre et al. (1952a) in Sweden. They recognized that pig pneumonia in Sweden was caused by an entity differing from swine influenza virus, and they suggested the name "enzootic virus pneumonia" for the disease. Fulton et al. (1953) in Canada, and Plowright (1953) in Kenya, succeeded in transmitting the disease by the intranasal or

intratracheal instillation of suspensions of pneumonic lung rendered bacteria-free by Seitz filtration. Rislakki (1953) in Finland, serially transmitted pneumonia in pigs with bacteria-free filtrates, but it is not clear whether he was transmitting swine influenza or virus pneumonia of pigs.

Thus by 1954, it had been clearly established that the chronic pneumonia of pigs, variously known as "infectious pneumonia of pigs", "virus pneumonia of pigs", or "enzootic virus pneumonia", could be serially transmitted in susceptible pigs.

Numerous attempts have been made to transmit the disease to laboratory animals. Gulrajani and Beveridge (1951) failed to transmit the disease to mice and ferrets. Fulton et al. (1953) were unable to infect guinea-pigs or mice by intranasal and subcutaneous injection of infective material. They also failed to transmit the disease to a Rhesus monkey by intranasal administration of the agent.

Wesslén and Lannek (1954) attempted to infect 5 week-old mice by the intranasal instillation of the swine enzootic pneumonia (SEP) 1 agent, isolated from chronic pneumonia in swine. The 1st and 9th cell culture passage levels were used as inoculum. The mice were killed on the 2nd and 7th days postinoculation, and examined for evidence of pneumonia, with negative results.

Surdan and Sorodoc (1960) found that mice, guinea-pigs, hamsters and rabbits were resistant to an agent isolated in

embryonated hens eggs from enzootic virus pneumonia of swine.

L'Ecuyer (1962) failed to infect 4 week-old mice by either intranasal or intraperitoneal administration of infective virus pneumonia of pigs lung suspension.

The propagation of the causative agent of virus pneumonia of pigs in embryonated hens eggs has been attempted many times during the past 15 years. Gulrajani and Beveridge (1951) failed to infect embryonated hens eggs by either allantoic-sac or chorio-allantoic membrane inoculation. Unsuccessful attempts at propagating the agent in chick embryos were also reported by Hjärre et al. (1952a), Plowright (1953), Penttinen and Rislakki (1953) and L'Ecuyer (1962).

Palyusik (1953) inoculated the chorio-allantoic membrane and the allantoic sac of 8 and 14 day-old embryonated hens eggs with a suspension of enzootic swine pneumonia lung. Pock-like lesions were observed on the chorio-allantoic membrane. The number of these lesions increased on subsequent passage. Second passage material was inoculated intranasally into susceptible pigs. A febrile reaction commencing on the 7th day and reaching a maximum temperature of 106.5 F on the 9th day was observed. Coughing was observed on the 21st day. The lesions found at necropsy were typical of enzootic swine pneumonia.

Lannek and Wesslén (1957) and Dinter et al. (1957) found that the agent recovered from swine enzootic pneumonia (SEP)

in cell cultures, grew readily when inoculated into the yolk-sacs of 5 to 7 day embryonated hens eggs. They believed yolk-sac inoculation to be a more sensitive method of isolating the SEP agent than cell culture inoculation.

Surdan and Sorodoc (1960) succeeded in propagating two strains of enzootic virus pneumonia agent by yolk-sac inoculation of embryonated hens eggs. They were able to transmit these agents through 12 and 13 serial passages respectively. Embryo deaths occurred between the 3rd and the 9th days post-inoculation. Hemorrhages, necrosis, hepatic hypertrophy and degeneration were seen in the dead embryos. Hemorrhage and edema of the yolk-sac membrane was observed. Pigs inoculated with 4th and 11th passage embryo tissues developed febrile reactions associated with catarrhal rhinitis and conjunctivitis between the 3rd and the 11th day. Bronchopneumonia with extensive lymphoid hyperplasia and mild suppuration developed in 80 per cent of the infected pigs. The infective agent was successfully reisolated in embryonated hens eggs from the lungs of the experimental pigs.

Bontschef (1961a, 1961b) isolated a strain of the virus pneumonia of pigs agent by pig inoculation, and after six consecutive passages, infected 5 to 6 day-old embryonated hens eggs by yolk-sac inoculation using a 15 per cent suspension of swine lung as inoculum. Embryo mortality occurred mainly between the 4th and 7th days postinoculation. Dead embryos showed petechial hemorrhages and stunting, membranes

were frequently edematous, and necrosis of the skin in the region of the head was occasionally observed. Pigs inoculated with embryo tissues from the 7th and 9th serial transfer, were killed 19 days postinoculation, and typical lesions of virus pneumonia of pigs were found. The agent was successfully reisolated in chicken embryos from a suspension of pneumonic lung and regional lymph-nodes.

L'Ecuyer (1962) and L'Ecuyer and Switzer (1963) attempted to infect embryonated eggs with two strains of the virus pneumonia of pigs agent. These were inoculated into the yolk-sac, chorio-allantoic membrane, and amniotic cavity of 6, 11 and 14 day-old embryonated hens eggs respectively. No mortality occurred, and no lesions were detected in the embryos. A suspension of pooled embryo tissues inoculated into susceptible pigs did not elicit gross or microscopic lung lesions.

By yolk-sac inoculation of embryonated eggs, Bakos and Dinter (1963) succeeded in isolating the swine enzootic pneumonia agent from 62.4 per cent of 120 pigs from enzootic pneumonia infected herds. The organism was isolated from 51.6 per cent of the nasal cavities and 41.6 per cent of the lungs examined. The agent was also successfully isolated from 18 out of 20 nasal swabs collected from clinically-ill pigs.

The isolation of a cytopathogenic agent in cell cultures from enzootic pneumonia in swine was reported by Wesslén and

Lannek in 1954. Swine lung and kidney cell cultures infected with a suspension of pneumonic lung showed cytopathic changes from the 3rd to the 10th day postinoculation. The agent was successfully passed through 11 serial transfers in swine lung cells. It was also grown in human lung cells, in bovine lung cells, and in bovine skin cells. The cytopathic changes observed in these cells resembled the changes seen in swine cell cultures. The agent was named the SEP 1 agent. Susceptible pigs were inoculated intranasally with material from the 4th and 10th serial passage levels, and killed after 12, 19 and 30 days. No pneumonic lesions were evident, but marked enlargement and edema of the bronchial and mediastinal lymph nodes was observed. The SEP 1 agent was successfully reisolated in cell cultures from the affected nodes. Dinter et al. (1957) later succeeded in propagating this agent in embryonated eggs.

Hjärre et al. (1954) attempted to propagate the enzootic pneumonia agent in cell cultures. Fibroblast and epithelial cells derived from swine kidneys were inoculated with the Sanda strain of enzootic pneumonia agent. In 3 out of 16 attempts a mild, slowly-developing cytopathic effect was observed. Subsequent attempts to reproduce these results failed, and the authors concluded that their results did not confirm those of Wesslén and Lannek (1954).

The results of further studies on the tissue culture propagation of the SEP agent were reported by Lannek and

Wesslén (1957). Four strains of the agent, SEP 1, SEP 7, SEP 26 and SEP 28, were successfully grown in swine kidney monolayers, and were administered to susceptible pigs by intranasal and intratracheal instillation. No macroscopic pneumonic lesions were evident at necropsy 3 weeks later, but 9 out of 15 pigs showed histological changes characteristically associated with enzootic pneumonia. Passage of the agents in suspended cell cultures of swine lung and kidney was then undertaken. Pigs inoculated intranasally with SEP agent passaged in this way developed macroscopically recognizable pneumonia and microscopic lesions identical with those of enzootic pneumonia. Intraperitoneal administration of the SEP agent resulted in the development of serositis as well as pneumonia. The reisolation of the SEP agent from lung tissues in these experiments was achieved without apparent difficulties. Switzer (1959) attempted to propagate the virus pneumonia of pigs agent in cell cultures. Primary swine lung and kidney cells, and serially passaged cultures of swine lung, kidney and nasal mucosa were inoculated with a field strain of the agent which had been passaged 2 to 7 times in respiratory disease-free pigs. No cytopathic effects were observed, and culture fluids from the 2nd passage in primary cell cultures did not induce pneumonia when inoculated into pigs.

L'Ecuyer (1962) and L'Ecuyer and Switzer (1963) succeeded in propagating two strains of the agent of virus pneumonia of

pigs in cell cultures. Primary cultures of swine kidney, lung, bone marrow, testicle, and chicken embryo kidney, as well as serial passage cell cultures of swine kidney, kidney-tumour, testicle, bone-marrow, bovine kidney and human cervical carcinoma (HeLa) were inoculated with the virus pneumonia agent. After 3 serial passages in HeLa cells typical gross and microscopic lesions were elicited in respiratory disease-free pigs inoculated intranasally and intratracheally with culture fluids. After 4 serial passages only microscopic lesions were produced, and after 6 and 11 passages no lesions were produced in test pigs. Typical gross and microscopic lesions were also produced in test pigs inoculated with culture fluids from the 2nd passage level of the agent in primary swine kidney cell cultures. Cytopathic effects were not observed in the infected HeLa or swine kidney cells. No specific changes were detected in cell sheets stained with Giemsa or acridine orange.

Goodwin and Whittlestone (1963a) and Betts and Whittlestone (1963) reported the successful propagation of the J strain of the agent of virus pneumonia of pigs in cell cultures of swine lung. Plasma-clot cultures were established from lung and nasal mucosa collected from a pig experimentally infected with the agent. Histological examination of this lung revealed the characteristic lesions of virus pneumonia of pigs. No macroscopic abnormalities were observed in the nasal cavity, but on histological examination of the nasal mucosa, basophilic

cytomegalic intranuclear inclusions were found. A cytopathic effect was observed in the migrating cells adjacent to the tissue fragments of the plasma-clot cultures from the 14th day onwards. The cells appeared granular and opaque and refractile particles were present in the cytoplasm. A pig inoculated intranasally and intratracheally with culture fluids collected from the lung cells on the 15th day developed typical macroscopic and microscopic lesions of virus pneumonia of pigs. The pig that received culture fluids from the nasal mucosa culture developed pneumonia. On histological examination of the nasal mucosa basophilic intranuclear cytomegalic inclusions were observed.

Pig lung monolayer cultures were then infected with fluids taken from plasma-clot lung preparations of virus pneumonia of pigs. A cytopathic effect resembling a mosaic was observed usually between the 3rd and 7th days postinoculation. The agent inducing the cytopathic effect was serially passaged 25 times. Susceptible pigs inoculated intranasally and intratracheally with 3rd and 4th passage tissue culture fluids developed moderately extensive lesions characteristic of virus pneumonia of pigs. No inclusions could be demonstrated in the nasal mucosa. One of 4 pigs inoculated with culture fluids from the 20th serial passage showed changes resembling resolving lesions of virus pneumonia of pigs.

Unsuccessful attempts were made to propagate the agent in

Maitland-type cultures, in pig kidney monolayer cultures, and by direct inoculation of pneumonic lung suspension onto pig-lung monolayer cultures.

A possible relationship between enzootic pneumonia and inclusion-body rhinitis was first suggested by Bakos et al. (1960). They found that susceptible pigs inoculated intranasally with the Sanda 15 strain of the enzootic pneumonia agent developed pneumonia and a catarrhal rhinitis associated with the presence of cytomegalic intranuclear inclusions in the glandular epithelium of the nasal mucosa. Obel (1961) found pneumonia in 14 out of 22 cases of inclusion-body rhinitis which she studied histologically. She described the condition as "alveolar cell pneumonia" and "interstitial lympho-histiocytic pneumonia". The intranasal instillation of a suspension of lung and nasal mucosa into 5 day-old pigs resulted in pneumonia and inclusion-body rhinitis. Goodwin and Whittlestone (1963a) and Betts and Whittlestone (1963) found inclusions in pigs inoculated with the J strain of the virus pneumonia of pigs agent. They were able to separate the pneumonia agent from the inclusion-body rhinitis virus by passage in pigs using pneumonic lung as the inoculum.

Goodwin and Whittlestone (1965) presented the results of experiments designed to determine the possible relationship between inclusion-body rhinitis, enzootic pneumonia and a type-XI pneumonia which they had previously described (1960,

1962, 1963b, 1964a, 1964b). They showed that the J strain of the enzootic pneumonia agent did not induce inclusion-body rhinitis, nor did the inclusion-body rhinitis agent produce gross pneumonia. They concluded that inclusion-body rhinitis and enzootic pneumonia were separate diseases. A suspension of lung and nasal mucosa that when inoculated intranasally into pigs produced both type-XI pneumonia and inclusion-body rhinitis, could be cleared of the pneumonia-producing factor by filtration through a Millipore filter of 300 m μ pore diameter. The filtrate could still be used to elicit inclusion-body rhinitis. Bordetella bronchiseptica was invariably found to be associated with the type-XI pneumonia, and the authors suggested it was the most likely cause of the disease. Duncan (1965) presented a detailed study of the pathogenesis of rhinitis and pneumonia induced by pure cultures of B. bronchiseptica. He pointed out the resemblance between the type-XI pneumonia and that induced with B. bronchiseptica, and also suggested that this bacterium was the probable cause of type-XI pneumonia.

The propagation of the swine enzootic pneumonia agent in cell-free media was reported by Bakos and Dinter (1963). They were able to grow the agent on "PPLO agar", and showed that the size of the colonies formed could be considerably enhanced when grown in symbiosis with staphylococci. The colonies resembled mycoplasmal colonies, and when grown in

symbiosis with staphylococci, measured up to 0.8 mm. in diameter. The recommended procedure for isolation of the agent was by yolk-sac inoculation of embryonated eggs, after which growth on "PPLO agar" was readily achieved. Direct isolation of the agent on the agar surface was seldom successful.

Goodwin and Whittlestone (1964c) produced enzootic pneumonia in pigs with a microorganism grown in media free from living cells. After initial propagation in pig-lung monolayer cultures, the J strain of enzootic pneumonia was inoculated into pig-lung monolayers which had been heated for 30 minutes at 56 C. They were able to grow the agent in this system until a dilution of 10^{-10} of the original inoculum was reached. Subsequently they changed their technique in favor of a boiled cell system. The fluids were removed from pig-lung monolayer cultures and replaced with phosphate-buffered saline. The tubes were then immersed in boiling water for 30 minutes, after which the saline was replaced with Hanks' balanced salt solution plus 20 per cent swine serum. The agent was successfully passaged 0 times in this medium at 3 or 4-day intervals. Pleomorphic organisms were present in large numbers in the cultures used for pig inoculation. Pigs killed 20 days after inoculation with these cultures had gross and microscopic lesions characteristic of enzootic pneumonia.

The J agent was subsequently passaged 8 times at 2-day intervals in the cell culture medium in the absence of cells. Pigs were inoculated intranasally with a culture which

represented a 10^{-15} dilution of the fluids originating from a passage in the boiled-cell medium. Gross and microscopic lesions of enzootic pneumonia were found at necropsy 20 days later. The authors concluded that the causal agent of the J strain of enzootic pneumonia had been grown in boiled cell medium, and also in medium free of cells.

The macroscopic and microscopic lesions of virus pneumonia of pigs have been described by many workers in different parts of the world. Pullar (1949a) in Australia, found that fibrinous pericarditis, pleuritis and peritonitis, as well as ulcerative gastritis and enteritis, were frequently associated with "infectious pneumonia". He reported that gross pneumonia was most often found in the right cardiac lobe, followed by the right apical and left cardiac lobes, and the left apical lobe. The ratio of occurrence was 4:2:2:1.

Hjärre et al. (1952a) in Sweden, described the histopathology of swine influenza and enzootic virus pneumonia (EVP) of pigs. During the early stages, both conditions were characterized by alveolar cell pneumonia with interstitial lymphohistiocytic infiltration and occasional giant cell formation. The tendency toward development of purulent bronchopneumonia was stronger in the EVP infections than in swine influenza.

Betts (1952) in England, noted that the lesions of virus pneumonia of pigs were usually confined to the antero-inferior portions of the lungs. Lesions were clearly

demarcated, plum-colored or sometimes greyish, in which case they resembled lymphoid tissue. Similar observations were made by Plowright (1953) in Kenya, and by Rislakki (1953) in Finland.

Dinter et al. (1954) described the pathogenesis of enzootic pneumonia infection. Lesions were usually confined to the anterior lobes and were characterized by early diffuse alveolar interstitial proliferation. This was often followed by the development of purulent bronchopneumonia which was postulated to be the result of secondary bacterial invaders.

Carter and Schroder (1956) described virus pneumonia of pigs in Canada and were able to transmit the disease to susceptible pigs. Lesions of bronchopneumonia with marked exudative changes, alveolar cell proliferation, peribronchial and peribronchiolar lymphoid hyperplasia, and neutrophilic infiltration were described.

Pattison (1956) presented a detailed account of the pathogenesis of virus pneumonia of pigs in field cases and in the experimentally produced disease. Gross lesions appeared on the 11th day postinoculation in the form of greyish-pink consolidated areas, most often in the anterior lobes. The outstanding microscopic lesion was extensive peribronchial, peribronchiolar and perivasculär lymphoid hyperplasia. Peribronchiolar and interalveolar accumulations of lymphocytes and macrophages were observed in pigs killed 24 hours postinoculation. In pigs killed at 7 days postinoculation distinct

lymphocytic cuffing of bronchioles and blood-vessels was evident. By the 11th day septal cell proliferation was observed, and the bronchiolar epithelium appeared hyperplastic. Few neutrophiles were encountered in either the natural or the experimental disease. Whittlestone (1957) described enlargement and edema of lymph nodes during the active phase of the disease. Hjärre (1957) described enzootic pneumonia as an alveolar cell pneumonia with alveolar epithelial hyperplasia. Hyperplasia of the peribronchial lymph nodes was a prominent lesion.

Underdahl and Kelley (1957) showed that lesions of virus pneumonia in experimentally infected pigs were markedly enhanced by migration of Ascaris suum larvae through the lungs. Similar enhancement of virus pneumonia of pigs lesions resulted from a concurrent infection with the lungworm, Metastrongylus elongatus (Mackenzie, 1963). Nelson (1961) failed in his attempts to recover the virus pneumonia of pigs agent from ascarid ova and larvae collected from pigs showing characteristic pneumonic lesions.

Urman et al. (1958) described the histopathological changes in experimentally produced virus pneumonia of pigs. On the 11th day postinoculation lymphocytic hyperplasia in the region of the blood-vessels and bronchioles was observed. From the 13th day onwards, a consistent histological picture characterized by marked perivascular and peribronchial cuffing and hypertrophy of the vessel endothelium was observed. There

was some increase in intraalveolar connective tissue with influx of lymphocytes and septal cells.

Trautwein (1960) described the histopathology of 79 field cases of virus pneumonia of pigs. Lesions were found predominantly in the anterior lobes, and were characterized by peribronchial, perivascular and interalveolar infiltration of lymphocytes, plasma cells and histiocytes. Alveolar exudates consisted of lymphocytes, plasma cells, leucocytes and alveolar cells. In advanced cases fibrosis and epithelialisation of the alveolar lining cells was observed. Proliferation and desquamation of the alveolar epithelium and lymphoid hyperplasia were also described as the prominent lesions of swine enzootic pneumonia in Japan by Konno et al. (1963).

Goodwin and Whittlestone (1963b) described the progressive histology of uncomplicated enzootic pneumonia. Alveolar cell pneumonia, catarrhal bronchiolitis and peribronchial and perivascular mononuclear-cell accumulations were followed by the progressive development of lympho-reticular hyperplasia with resolution of the alveolar cell pneumonia. Field cases of the disease were frequently complicated by purulent bronchiolitis.

Hänichen (1964) described and discussed the changes in the bronchial and alveolar epithelium in swine enzootic pneumonia, with emphasis on the question of whether or not these changes could serve as precursors of tumors in the lungs. During the early stages of the disease, degenerative changes

were observed in the bronchial epithelium. These were followed by epithelial hyperplasia which was considered to be the normal regenerative process, and not early signs of neoplasia. Alveolar cell proliferation and desquamation, as well as peribronchial and perivascular lympho-histiocytic infiltration was observed, followed by chronic interstitial pneumonia with fibrosis of the alveolar septa.

Filtration techniques have been used to clear virus pig pneumonia inocula of bacterial contaminants, and also in attempts to determine the size of the causative agent. Gulrajani and Beveridge (1951) were able to reproduce the disease with an agent which passed Gradocol membranes of 0.8μ but not 0.56μ average pore diameter. The passage of the agent through filters of 0.8μ pore diameter has also been reported by Goodwin and Whittlestone (1963a, 1964c).

Fulton et al. (1953) and Plowright (1953) were able to reproduce the characteristic lesions of virus pneumonia in pigs with inocula which had been cleared of bacteria by filtration through Seitz asbestos pads. Momberg-Jørgensen (1938) reproduced pneumonia with an agent passed through filters with an average pore diameter of 0.94μ .

Wesslén and Lannek (1954) were able to infect cell cultures with a strain of the SEP agent which had been passed through Zsigmondy cellulose filters with an average pore diameter of 0.75μ . Lannek and Wesslén (1957) subsequently showed that the SEP agent could pass Zsigmondy filters with

average pore diameters of 0.3μ (maximum pore size 0.5μ).

Switzer (1956) was able to produce pneumonia and turbinate atrophy in pigs inoculated intranasally with turbinate suspensions filtered through Selas No. 015 filters and cellulose membranes of 0.75 to 1.0μ pore size. Carter and Schroder (1956) filtered suspensions of ground lung through Seitz clarifying pads and Selas No. 02 candles, and reproduced typical lesions of virus pig pneumonia in susceptible pigs.

Betts (1952) and Betts and Beveridge (1952) performed filtration trials to determine the size of the agent of virus pneumonia of pigs. They concluded that a virus of not less than 200-250 $\text{m}\mu$ diameter was the causative agent of the disease. Several attempts were made by these workers to detect Mycoplasma in smears of lung tissue, but none was found.

The successful visualization of an agent believed to be the cause of swine enzootic pneumonia was reported by Lannek and Wesslén (1955). The SEP 1 agent was propagated in cell cultures of bovine embryonic skin, which were fixed and stained with a number of different stains at various intervals after inoculation. In haematoxylin-eosin stained preparations, eosinophilic cytoplasmic masses were observed in a large number of cells on the 2nd day postinoculation. These masses varied considerably in size, sometimes filling the cytoplasm. Granular bodies resembling hyaline drops occurred in the cytoplasm in the region of the inclusions. Cytoplasmic degeneration was usually complete by the 8th day. Nuclear changes

were studied in cells stained by the Feulgen technique, and were found to commence later than the cytoplasmic changes. Pyknosis was followed by clumping of the chromatin and disappearance of the nuclear membranes, leaving the chromatin balls free in the degenerated cytoplasm. Large numbers of distinctly stained intracytoplasmic particles were seen at 800X magnification in cells stained by Taniguchi-Gutstein's methyl violet, or Gutstein's modification of Herzberg's technique. These bodies were readily demonstrable in unstained preparations examined by phase contrast microscopy. Castaneda and Machiavello staining did not reveal a relationship between these bodies and rickettsiae. No cyclic development resembling the growth pattern of the psittacosis agents was observed. The nature of the particles was not understood, but it was suggested that they might be the enzootic pneumonia agent.

Dinter (1957) was able to demonstrate particles in yolk-sac membranes of embryonated eggs inoculated with the SEP agent. When stained with Giemsa the particles resembled those seen in cell cultures infected with the agent.

Whittlestone (1957) described extracellular pleomorphic organisms (P.O.) in Giemsa-stained touch preparations of fresh pneumonic lung from experimentally produced or field cases of enzootic pneumonia. The organisms resembled Mycoplasma tinctorially and morphologically, and were not encountered in other types of pneumonia. Betts and Whittlestone (1963) and

Goodwin and Whittlestone (1963a) described particles resembling P.O. in cell cultures prepared from turbinate mucosa and lungs from pigs experimentally infected with the J strain of enzootic pneumonia. They were subsequently able to reproduce the disease with fluids from the cell cultures. The P.O. found in the cell cultures resembled those seen in touch preparations from pneumonic lungs, and were believed to play an essential role in the disease. It was suggested that the P.O. were likely to fall into the Mycoplasma group.

Goodwin and Whittlestone (1963b) defined the diagnostic criteria for enzootic pneumonia, and put considerable emphasis on the presence of P.O. in touch preparations from fresh lung lesions. In their successful attempt to propagate the agent in cell-free media, Goodwin and Whittlestone (1964c) found P.O. present in large numbers in the inocula used to reproduce the disease in susceptible pigs.

In several excellent histological studies of virus pig pneumonia no mention was made of the occurrence of pleomorphic organisms in lung lesions (Pattison, 1956; Hjärre, 1957; Urman et al., 1958; and Hänichen, 1964).

The antibiotic sensitivity of the virus pneumonia of pigs agent was investigated by Betts and Campbell (1956). The in vitro, prophylactic and curative actions of sulphamezathine, penicillin, streptomycin, chloromycetin, chlortetracycline, oxytetracycline and tetracycline were determined in a series of experiments using the M.R. strain of the agent. None of the

drugs tested had any effect on established lesions. Penicillin, streptomycin and chlortetracycline had no demonstrable in vitro effect on the virus. The other drugs were not tested for in vitro effect. Sulphamezathine, penicillin, chloramphenicol and chlortetracycline feed supplement had no prophylactic action. The intravenous administration of chlortetracycline and oxytetracycline exerted a marked prophylactic effect. Slight protection resulted from the parenteral administration of streptomycin commencing 5 hours before exposure to the agent, and continuing for seven days. The in vitro resistance of the agent to penicillin and streptomycin has also been reported by Betts (1952), Betts and Beveridge (1952), Fulton et al. (1953), Penny (1954), Wesslen and Lannek (1954) and Lannek and Börnfors (1956).

Penny (1954) and Pugh (1956) reported that chloramphenicol treatment of virus pneumonia infected pigs resulted in reduced coughing and increased weight gains. It was suggested that this was the result of therapeutic control of secondary bacterial invaders and not of the primary pathogen.

Wesslen and Lannek (1954) showed that the SEP agent was highly sensitive to chlortetracycline and oxytetracycline in vitro. This finding was in agreement with the earlier observations of Betts (1952) and Betts and Beveridge (1952).

Börnfors and Lannek (1955) investigated the therapeutic effect of sulphamezathine, sulphanilamide, penicillin, chlortetracycline and oxytetracycline on field cases of enzootic

pneumonia. The criteria by which they measured therapeutic effect were growth, feed consumption, feed conversion rate and radiological examinations of the lungs. With the exception of sulphanilamide, all the drugs were of therapeutic value on the basis of improved growth, feed consumption and feed conversion, but no change in the extent of pneumonic lesions was observed. They proposed that the beneficial effect was probably the result of suppression of secondary bacterial invaders.

Lannek and Börnfors (1956) found that tetracycline and oxytetracycline when given per os at dosage levels ranging from 10 to 20 mg./kg. had a marked prophylactic effect on artificially induced enzootic pneumonia infection. Goret et al. (1960) showed that tetracycline HCl in the feed protected susceptible pigs from infection, and stimulated growth in infected pigs. Prophylactic effect could be achieved by parenteral administration of the drug, but administration via the feed was considered to be the route of choice. It was suggested that resistance to reinfection was due to the continuous presence of the virus in the pig, a phenomenon resembling premunition.

An attempt was made by Betts and Beveridge (1952) to demonstrate antibodies in the convalescent sera of pigs infected with virus pneumonia of pigs. No neutralizing antibodies could be demonstrated. They also made an unsuccessful attempt to vaccinate pigs with a suspension of pneumonic lung. Penttinen and Rislakki (1953), using haemagglutination-

inhibition and complement fixation tests, failed to demonstrate antibodies in sera from pigs with virus pneumonia. Unsuccessful attempts to demonstrate neutralizing antibodies were also reported by Hjärre et al. (1951, 1952a), Wesslén and Lannek (1954) and Surdan and Sorodoc (1960).

Lannek and Börnfors (1957) demonstrated that pigs which had recovered from experimentally induced enzootic pneumonia, were resistant to challenge with the same strain of the agent. They proved that age was not the factor responsible for the resistance by inoculating ten pigs of the same age and size, all of which developed the disease. Subsequently the same authors (Börnfors and Lannek, 1958) demonstrated that resistance to enzootic pneumonia could not be induced by the intramuscular administration of the agent.

In vitro neutralization of the SEP agent was reported by Dinter et al. (1957). Antiserum was prepared in rabbits, and neutralization tests were successfully performed in cell cultures and in embryonated eggs. Bakos and Dinter (1963) successfully repeated their attempts at neutralization of the agent, and were also able to develop an indirect fluorescent antibody procedure and an agar-gel precipitation test.

Howell and Gordon (1954) reported the demonstration of cold agglutinins to human group O erythrocytes in pigs which were subsequently found to have the typical lesions of virus pneumonia of pigs. In a series of controlled experiments Fontaine et al. (1959) confirmed these findings, but also

demonstrated the antibodies in sera of swine free of virus pneumonia of pigs.

Gulrajani and Beveridge (1951) showed that the infectivity of the virus pneumonia of pigs agent was not destroyed by storage at -20 C or in glycerol at 0 C for up to 55 days. Betts (1952) found that the agent survived for at least 24 hours in a buffered solution at room temperature. It was later reported that the agent was rendered noninfective for pigs by exposure to 20 to 22 C for 72 hours, or 37 C for 8 hours (Betts and Whittlestone, 1963).

Betts (1952) has been able to recover the virus pneumonia of pigs agent from a pig infected 66 weeks previously, and from several pigs infected 21 weeks previously. Hjärre et al. (1952a) found that lungs from pigs infected 4 months previously were still infective when inoculated into susceptible pigs.

Human Primary Atypical Pneumonia

The term "Primary Atypical Pneumonia" has been used since World War II to designate a nonbacterial, noninfluenzal lower respiratory tract syndrome in man, which occurred in pandemic form in areas of troop concentration (Reimann, 1964).

Meiklejohn (1943) demonstrated significant titers of cold agglutinins for human type O erythrocytes in a large proportion of cases of primary atypical pneumonia. Similar titers were not observed in other respiratory diseases. Thomas et al.

(1943) showed that streptococcal MG agglutinins were frequently present in sera of patients recovering from the disease. Eaton et al. (1944) reported the isolation of an agent from patients with primary atypical pneumonia. They were able to propagate the agent in embryonated eggs, and could then infect hamsters and cotton rats by the intranasal instillation of chicken embryo tissues. Filtration experiments were performed using collodion membranes, and on the basis of the results, the size of the agent was estimated to be between 180 and 250 $\text{m}\mu$ (Eaton et al., 1945). Eaton (1950) later showed that the agent was highly sensitive to chlor-tetracycline, but that chloromycetin had little effect under the same experimental conditions.

A study of the localization of the Eaton agent in chicken embryos using an indirect fluorescent antibody technique was reported by Liu (1957). The agent was found to multiply exclusively in the cytoplasm of the bronchiolar and air-sac lining epithelium. Marmion and Goodburn (1961) and Clyde (1961) were able to visualize small coccobacillary bodies situated extracellularly in stained preparations of chick embryo bronchial epithelium and infected cell cultures.

Chanock et al. (1962a) succeeded in propagating the Eaton agent on horse serum agar medium. On the basis of the morphologic and staining properties of the colonies, and their requirement for serum, it was concluded that the organism was a member of the genus Mycoplasma. Using a fluorescent

antibody staining technique they were able to show that the Eaton agent was antigenically distinct from three human oral, and four human genital strains of Mycoplasma as well as a number of other mammalian, avian and cell cultures strains. Subsequently they propagated the agent in broth medium supplemented with horse serum and yeast extract, and prepared an antigen suitable for use in a complement fixation test (Chanock et al., 1962b).

Somerson et al. (1963) proposed the name Mycoplasma pneumoniae for the agent of primary atypical pneumonia. They showed that the agent produces a hemolysin capable of lysing guinea-pig erythrocytes and suggested that this property might be useful in identification of M. pneumoniae isolates. Clyde (1963) found that hemolysis was most striking when sheep or guinea-pig erythrocytes were used, but human, horse and rabbit erythrocytes were also satisfactory. Kerr et al. (1964) developed a rapid plate agglutination test for the diagnosis of M. pneumoniae infection. Dowdle and Robinson (1964) developed a specific indirect hemagglutination test which was as sensitive as the complement-fixation test and more sensitive than the fluorescent antibody techniques.

Hilleman et al. (1962) surveyed the occurrence of primary atypical pneumonia in various age groups, and found that this disease was the principal causative agent in adults hospitalized for respiratory disease between 1953 and 1960.

The resemblance between primary atypical pneumonia of

man and virus pneumonia of pigs was recognized by Betts and Beveridge (1952). They suggested that size and antibiotic sensitivity indicated a possible relationship between the virus pneumonia of pigs agent and the grey-lung virus of mice, psittacosis agent, Mycoplasma and the primary atypical pneumonia agent.

The resemblance between the lesions of virus pneumonia of pigs and grey-lung disease of mice was discussed by Pattison (1956). The lesions encountered in the late stages of the mouse pneumonia very closely resembled the lesions of virus pneumonia of pigs.

Betts and Campbell (1956) discussed and compared the antibiotic sensitivity of the Eaton agent, the grey-lung virus of mice, and the agent of virus pneumonia of pigs. Chlortetracycline had a marked prophylactic and curative action on the Eaton agent and on the grey-lung virus, but was effective only in the prophylaxis of virus pneumonia of pigs. Chloromycetin appeared to have no effect on the three agents.

In a survey of the lungs of 700 market age pigs, Young and Underdahl (1960) encountered a pneumonia that grossly resembled virus pneumonia of pigs. On microscopic examination it proved to be an interstitial pneumonia distinctly different from virus pneumonia of pigs and was said to resemble primary atypical pneumonia of man. Roberts et al. (1962) encountered one case of interstitial pneumonia in a survey of the microscopic lesions present in 86 pneumonic swine lungs. They were

able to produce lesions resembling virus pneumonia in pigs inoculated with material from this case.

Ultrastructure of Mycoplasma

Early electron microscopic studies on members of the Mycoplasmatales (Reagan et al., 1951, 1953) revealed extensive filament formation in cultures of unidentified avian Mycoplasma strains. Thin-sectioning techniques were not used, and thus no description of the internal structure of these agents was given. Morowitz et al. (1952) in a study of the chemical composition and submicroscopic morphology of Mycoplasma gallisepticum, showed that the cells of the agent were generally spherical with an average diameter of 250 m μ . Occasional cells with diameters as large as 500 m μ and as small as 150 m μ were encountered.

Shifrine et al. (1962) described the various morphological forms of M. gallisepticum as seen with the electron microscope during colonial growth on solid media. Three distinct forms of growth were observed. The early stage consisted of small hexagonal cells, referred to as elementary cells, measuring from 100 m μ to 500 m μ in size. Large hexagonal cells which the authors called platocytes were then observed. These were followed by the development of the third form called exoblasts. No sectioning of the agents was reported, and no internal structure was described.

The electron microscopic examination of ultra-thin

sections of Mycoplasma colonies and of cell cultures infected with Mycoplasma was reported by Edwards and Fogh (1960). They found that the organisms generally were ovoid measuring on an average 250 by 300 $\text{m}\mu$. The Mycoplasma lacked a cell wall, but possessed a plasma membrane. The cytoplasm was peripheral and contained large nucleoids and granular matrices. Threadlike filaments believed to represent chromosomes were described. Various stages of budding and multi-nuclear division were observed. The entry of the Mycoplasma into the host cell commenced with apposition of plasma membranes followed by microvillus formation, swelling of endoplasmic reticulum, distortion of mitochondria, vacuole formation, cytoplasmic granulation and then breakdown of the membranes. The Mycoplasma in the cell were usually, but not always, situated within cytoplasmic vacuoles.

Freundt (1960) presented electron micrographs of thin sections of Mycoplasma mycoides grown in broth cultures, showing elementary bodies with dense cytoplasm and delicate cytoplasmic membranes.

Van Iterson and Ruys (1960) used the shadow-casting and ultra-thin sectioning techniques to study the morphology of Mycoplasma hominis, Mycoplasma salivarium and Mycoplasma fermentans. They found that colonies of all three species contained coccoid elements as well as the usual vesicular elementary bodies. In section the coccoid bodies resembled bacteria very closely, being much more electron-dense than

the vesicles, and possessing a distinct cell wall, dense cytoplasm and a well-defined nuclear structure. In sections they averaged $270\text{ }\mu$ in diameter which was approximately the same size as the Mycoplasma vesicles. A very close association between the two forms was demonstrated, and the authors suggest that the Mycoplasma of human origin may represent the L-phase variant of minute coccoid bacteria.

An attempt was subsequently made by the same authors (Ruys and van Iterson, 1961) to demonstrate the coccoid bacteria in Mycoplasma of avian origin. In all seven strains of M. gallisepticum that they examined they were able to demonstrate two distinct colony types. Small granular colonies 15 to $40\text{ }\mu$ in diameter were most numerous, but a few large flat homogeneous translucent colonies 50 to $250\text{ }\mu$ in diameter were invariably present. Subcultures from each type of colony gave rise to both types, and separation of the two types by filtration could not be achieved. Electron microscopic examination of ultra-thin sections of the small colonies revealed the typical vesicular mycoplasmal morphology. The large colonies consisted of electron-dense bodies which in their compactness and internal organization resembled the small coccoid bacteria found in the human strains of Mycoplasma. No bacterial cell walls could be demonstrated however, and it was concluded that these agents represented an intermediate stage between Mycoplasma and bacteria.

Domermuth et al. (1964a, 1964b) described the ultrastructure of 19 strains of Mycoplasmatales grown on solid media. In all the strains examined a triple-layered limiting cell membrane of 75 to 100 Å thickness was observed. This membrane appeared to be symmetrical in some strains and asymmetrical in others. The cytoplasmic components of the cells, such as ribosomes and nuclear regions with DNA-like strands, closely resembled those found in bacteria. In the JA strain of M. gallisepticum the ribosomes were arranged in a "corn-cob" pattern, a phenomenon not seen in other strains.

The size of the "average Mycoplasma cell" as measured on electron micrographs was 500 to 700 μ , a dimension which was found to be remarkably uniform between the different strains. The external elementary bodies had an average size of only 130 to 150 μ . The small elementary bodies were very electron-dense in contrast to the larger cells which on occasion contained areas of lesser density or appeared completely empty. It was suggested that the large empty forms represented a non-viable involution form of the agent.

Homogeneous spherical condensations 100 to 200 μ in diameter, with or without limiting membranes were often encountered within the cytoplasm of the larger Mycoplasma cells. These structures were usually located at the periphery of the cells, in or near filamentous protrusions of the limiting membrane. It was suggested that the mycoplasmal filaments might be the site of elementary body production and liberation.

In an electron microscopic study Dmochowski et al. (1964) using the negative staining and thin-sectioning techniques were able to demonstrate Mycoplasma associated with human leukemia. They discussed and compared the morphology of known Mycoplasma and the organisms found in the blood and lymph nodes of leukemic patients.

Ultrastructure of Psittacosis Agents

The agents of the psittacosis-lymphogranuloma-trachoma (PLT) group possess characteristics linking them with both the viruses and the bacteria. They differ from the viruses in their size, complex developmental cycle, antibiotic sensitivity and the possession of DNA, RNA, muramic acid and certain enzymes. Like the viruses, they are obligate intracellular parasites, and in some of them the DNA replication resembles that of the viruses. (Armstrong et al., 1963; Erlandson and Allen, 1964; Tanami et al., 1961.) The developmental cycle of the psittacosis agent was described by Bedson and Bland (1932), who also described the morphology of the agent as seen through the light microscope.

Gaylord (1954) performed the first definitive electron microscopic study of the ultrastructure of a psittacosis agent. In ultra-thin sections of chorio-allantoic membranes infected with the Cal 10 strain of meningopneumonitis agent he was able to demonstrate round to oval structures of varying sizes, presumably different stages in the life cycle of

the agent. The particles identified as elementary bodies were electron-dense, frequently with dark central granules, and measured from 250 to 300 $\text{m}\mu$ in diameter. Intermediate forms, less dense than the elementary bodies, and measuring from 300 to 400 $\text{m}\mu$ were frequently seen. Larger forms referred to as "circles" were commonly encountered. These structures were much less electron-dense, measured from 400 to 600 $\text{m}\mu$ in diameter, occasionally contained dense granules and sometimes appeared to be dividing. They were contained within a single limiting membrane which often appeared to be ruptured. It was concluded on morphological evidence that the agents divided by binary fission or by multiple endosporulation.

Tajima et al. (1957) described the structure and intra-cellular development of a bovine PLT group agent in mouse lung, and of the Cal 10 strain of meningopneumonitis in the chorio-allantoic membrane. Various developmental forms were observed with both agents, and the developmental sequence of these forms was followed. Twenty-four hours after inoculation an amorphous granular matrix was observed in the cells. Incomplete membranes appeared to condense on the surface of the matrices, finally enclosing them forming the "large" forms. Reduction in size and the formation of central granules results in what was referred to as the "intermediate" form. Further reduction in size and increase in density gave rise to the elementary bodies which were considered to be the terminal

stage of development. All of the above forms were enclosed within "inclusion bodies" within the cytoplasm.

Page et al. (1961) compared the structural features of 13 strains of avian and mammalian PLT agents. By using the shadow-casting technique they were able to demonstrate the outer membranes of the agents, and both spherical and polygonal forms of the agents. The diameters of the organisms varied from 280 to 980 μ .

A light and electron microscopic study of the morphology and development of the trachoma agent in human conjunctiva and in the yolk-sac of embryonated eggs was reported by Mitsui et al. (1962). They showed that the agent first developed plaques, then initial bodies, and finally elementary bodies. Typical elementary bodies consisted of an electron-dense central nucleoid surrounded by viroplasm and a limiting membrane. Other electron-dense, less clearly defined elementary bodies with irregular boundaries were described.

Armstrong and Reed (1964) described the morphology and early development of the lymphogranuloma-venereum (LGV) agent in HeLa cell cultures. The elementary bodies of LGV resembled those of psittacosis and trachoma. A central electron-dense core consisting of closely packed granular material contained a localized fibrillar nucleoid. The entire electron-dense central region was surrounded by a less dense region and single limiting membrane. The diameter of the elementary bodies was about 325 μ .

The process of attachment and entry of the agent into the cell was described in detail. A comparison was made between fixation of cells with Palade's solution, potassium permanganate and Kellenberger's method, and it was concluded that the results obtained using the last-named technique most nearly represented the true morphology of the agents. On the basis of morphology and development it was suggested that the psittacosis group may have evolved by ecological specialization of free-living bacteria-like ancestors.

Erlandson and Allen (1964) described the ultrastructure of intracellular, crude and purified meningopneumonitis agent. Using both the thin-sectioning and negative staining techniques, they were able to describe 2 types of particles; the relatively large initial bodies and the small dense elementary bodies. Initial bodies were bounded by a 70 Å unit membrane, and measured from 400 to 590 μ in diameter. The bodies were made up of a fibrillar matrix in which membranous "whorls" resembling fingerprints were seen. The elementary bodies were round to ovoid, and measured 260 to 310 μ in diameter. A very dense central nucleoid was separated from the 70 Å limiting membrane by a faint ground substance. "Whorls" were occasionally encountered in the elementary bodies, and in some cases appeared to be continuous with the limiting membrane. It was suggested that these structures could be analogous to the mesosomes of bacteria.

Mitsui et al. (1964) studied the development and morphology of the trachoma agent in the yolk-sac of embryonated eggs. Reticulated matrices composed of 100 Å filaments were described as the precursors of the viral forms. These matrices then became surrounded by double membranes to form the "giant bodies" (plaques), measuring 1 to 5μ in diameter. Membrane-bound inclusion cavities developed around the "giant bodies" which decreased in size to become "large bodies" (initial bodies) measuring 0.5 to 1μ in diameter. The elementary bodies which developed after the "large bodies", measured 300 to 500 $\text{m}\mu$ in diameter, and consisted of an electron-dense nucleoid composed of coiled filaments, a slightly less dense viroplasm and a single limiting membrane. The elementary bodies were believed to arise from the earlier developmental forms by a process other than binary fission.

In an electron microscopic study of latent psittacosis virus in McCoy cells, Kajima et al. (1964) were able to demonstrate three well-defined morphological stages; an initial body stage, an intermediate body stage, and the mature elementary body forms. With the aid of acridine-orange staining they were able to demonstrate that only the elementary body form of the virus was infectious. The initial bodies corresponded with the RNA-staining stage as observed in acridine orange stained preparations. The mature elementary bodies measured 250 to 300 $\text{m}\mu$ in diameter, and characteristically

consisted of a dense nucleoid separated by a space from a distinct virus membrane. Binary fission of the mature virus particles was not observed.

MATERIALS AND METHODS

Virus Pneumonia of Pigs Agent

The agent of virus pneumonia of pigs used throughout these experiments was strain No. 11 (L'Ecuyer, 1962). This strain was isolated from a pig originating from a large herd with a continuing chronic pneumonia problem. The pig from which the strain was isolated had gross consolidation of the apical and cardiac lobes of the lungs. Intranasal inoculation of respiratory disease free pigs with the agent consistently resulted in well-demarcated, reddish-grey pneumonic lesions, generally involving the cardiac, apical and intermediate lobes. The lesions occurred as scattered foci of consolidation or involved entire lobes. The microscopic lesions characteristically elicited by this agent were peribronchiolar and perivasculär lymphoid hyperplasia, marked alveolar interstitial thickening, and alveolar exudation of septal cells, mononuclear leucocytes and in early cases, neutrophiles.

An apparent increase in virulence of the agent was observed after serial passage in pigs. During the early passages, lesions were elicited in 30 to 60 per cent of inoculated pigs. After 7 serial passages, lesions were usually present in 80 to 100 per cent of inoculated pigs. During the course of the experiments the agent was serially passaged from the 3rd to the 10th passage level in pigs.

Lesions from experimentally infected pigs were

aseptically removed, cut into small pieces, and stored in rubber-stoppered vials in a freezer cabinet at -30 C or in a dry ice and alcohol chest at -65 C. Routine bacteriological examination of the infective lung material was performed by inoculation of the following media: 1. horse-blood agar plates streaked with a micrococcal nurse colony, 2. modified MacConkey's agar (Ross, 1965), and 3. beef heart infusion broth with hemoglobin, swine gastric mucin and turkey serum (Ross and Switzer, 1963). This strain of the virus pneumonia of pigs agent has consistently been shown to be free of Mycoplasma hyorhinis, Mycoplasma granularum, Haemophilus spp., Pasteurella multocida, Bordetella bronchiseptica and Streptococcus spp. It was shown to be free of swine influenza virus by inoculation of embryonated hens eggs and intranasal inoculation of mice. No cytopathogenic viruses were isolated in primary swine kidney cell cultures inoculated with the agent.

Suspensions of infective lung used for inoculation of pigs, cell cultures, artificial media, mice and embryonated eggs were prepared by grinding the tissue in a glass tissue grinder with physiological saline, or with Dulbecco's phosphate buffer (Dulbecco and Vogt, 1954) plus 10 per cent calf serum. A 10 per cent suspension prepared in this way was centrifuged at 2000 G for 10 to 15 minutes to sediment cellular debris. The supernatant fluid was either used

directly, or filtered through cellulose membranes¹ of 800 m_μ APD prior to inoculation.

Experimental Pigs

The experimental pigs were procured from two sources. Most of them were from the respiratory disease-free herd maintained at the Iowa Veterinary Medical Research Institute. This herd was established in 1951 with surgically derived breeding stock, and all subsequent introductions into the herd have been surgically derived. The experimental pigs were naturally farrowed and raised with the sows until weaning. A few experiments were performed using surgically derived, colostrum deprived pigs reared in isolation in the Department of Veterinary Hygiene, Iowa State University.

The clinical, pathological and bacteriological examinations performed annually on 300 to 400 pigs from this herd confirm its continued respiratory disease-free status. Management and isolation programs were strictly supervised, and all rations were prepared free of antibiotics or other drug additives.

Experimental pigs were housed in strict isolation for the duration of each experiment and were in most cases allotted to units by an approved randomization procedure. The ages of the pigs ranged from 3 to 10 weeks. One to 2 ml. of inoculum was

¹Millipore Filter Corporation, Bedford, Massachusetts.

administered intranasally by injection through a rubber urethral catheter inserted into the nasal passage to a depth of approximately 3 inches. Two, 3 or 4 inoculations were given at 2 to 3 day intervals.

Pigs were necropsied after observation periods ranging from 3 to 8 weeks. The usual procedure was to electrocute and then exsanguinate the pigs, but for the collection of specimens for electron microscopic examination, barbiturate anaesthesia was used. All animals were examined for gross lesions and specimens were collected for histological examination and aseptically for bacteriological procedures. Routine inoculations of horse-blood agar plus nurse colony, modified MacConkey's agar and beef heart infusion broth, as described earlier, were made.

Propagation Experiments

Cell cultures

The 3 types of cell cultures used in these investigations were primary swine kidney cells, primary swine lung cells, and serial passage swine lung cells. All cells were incubated at 37 C in a stationary position.

Glassware preparation The distilled water used in washing of glassware and stainless steel utensils had been passed through a mixed-bed resin ion exchange column, and shown to have a total conductivity equivalent to less than 0.1 parts per million NaCl.

The dirty glassware was immersed in 0.06 per cent Micro-solve¹ detergent and mechanically brushed to remove adherent substances. It was then heated to approximately 65 C in fresh detergent solution, rinsed in three changes of ion exchange water, heated to over 65 C in ion exchange water, and then inverted in racks to dry. The final rinse had a conductivity of less than 0.3 parts per million NaCl equivalents. After appropriate wrapping the clean glassware was sterilized at 160 C dry heat for 3 hours.

Preparation of media Ion exchange water was used in the preparation of all culture media and other solutions used in the cell culture procedures. Three balanced salt solutions were used. A modification of Madin's balanced salt solution (1957) was prepared as indicated below.

NaCl	7.0	gm.
KCl	0.4	gm.
CaCl ₂ •2H ₂ O	0.26	gm.
MgSO ₄ •7H ₂ O	0.2	gm.
NaH ₂ PO ₄ •H ₂ O	0.144	gm.
Dextrose	1.0	gm.
Lactalbumin (enzymatic) hydrolysate (LH)	5.0	gm.
Phenol red, 0.2 per cent	5.0	ml.
Ion exchange water	1000.0	ml.

¹Microbiological Associates, Inc., Bethesda, Maryland.

The solution was placed in Erlenmeyer flasks with cotton plugged air-vents penetrating the rubber stoppers, and autoclaved at 10 pounds of pressure as suggested by Hancock et al. (1959) for 20 minutes. Earle's balanced salt solution (1943) with 0.5 per cent LH, and Hanks' balanced salt solution (1948) with 0.5 per cent LH, were also used. Both these media were autoclaved at 10 pounds pressure as described for Madin's balanced salt solution.

Ten per cent bovine fetal serum (BFS), 10 to 15 per cent calf serum (CS), or 20 per cent swine serum (SS) was added to the balanced salt solutions, depending on the cell type being grown. Culture media used for propagation of primary swine kidney cells contained 200 units of penicillin and 100 micrograms of streptomycin per ml.

Primary swine kidney cells Kidneys were aseptically removed from 1 day-old to 8 week-old pigs from the respiratory disease-free herd maintained at the Iowa Veterinary Medical Research Institute. They were finely minced with scissors, washed thoroughly with the balanced salt solution A (BSA) described by Marcus et al. (1956), and trypsinized with 0.33 per cent Trypsin 1:250¹ in BSA by a modification of the continuous-flow digestion system described by Melnick (1955). Digested cells were collected in a centrifuge bottle chilled at 4 C, and when digestion was complete, centrifuged at 1000

¹Difco Laboratories, Detroit, Michigan.

RPM for 5 minutes. The supernatant fluid was discarded, and the cells were washed and centrifuged twice at 600 RPM for 3 minutes using the complete growth medium consisting of Madin's balanced salt solution with LH, 10 per cent CS and antibiotics, adjusted to pH 7.0 with 7.5 per cent sodium bicarbonate. The packed cells were then resuspended in the complete growth medium at a dilution of 1:300, and dispersed in 1.5 ml. amounts into 16 x 150 mm. Kimax¹ tubes, in 2.0 ml. amounts into 16 mm. Leighton tubes containing 11 x 22 mm. cover-slips, and when required, into tissue culture flasks. The tubes were placed in racks and incubated in a stationary position for 3 to 4 days at which time the growth medium was replaced with 2.0 ml. maintenance medium consisting of Earle's balanced salt solution with LH, 10 per cent CS and antibiotics. The cells were again incubated in the stationary position until required for inoculation.

Primary swine lung cells Lung tissue was aseptically removed from respiratory disease-free pigs or from pneumonic lesions in experimentally infected pigs. The tissue was placed in a sterile beaker, finely minced with scissors, and then transferred to a sterile Erlenmeyer flask containing a teflon-coated magnetic stirring bar. After one rinsing with BSA it was trypsinized in a 0.33 per cent solution of Trypsin² 1:250

¹Kimble Glass Company, Toledo, Ohio.

²Difco Laboratories, Detroit, Michigan.

in BSA at 4 C for 18 to 24 hours. The flask was placed on a magnetic stirrer and trypsinization was allowed to continue for 2 to 3 hours at room temperature. The cell suspension was then strained through 4 layers of sterile gauze, placed in a centrifuge tube and centrifuged at 800 RPM for 10 minutes. The supernatant fluid was decanted and the cells were resuspended in Madin's balanced salt solution with LH and sedimented by centrifugation at 600 RPM for 3 minutes. The final cell suspension was made in Madin's balanced salt solution plus LH and 10 per cent BFS without antibiotics, giving a final cell dilution of 1:200. The cell suspension was dispersed in 1.5 ml. amounts into 16 x 150 mm. culture tubes, in 2.0 ml. amounts into 16 mm. Leighton tubes containing 11 x 22 mm. cover-slips, or into tissue-culture flasks as the occasion demanded. The culture medium was renewed on the 3rd or 4th day and cell sheets were usually confluent by the 5th day.

Serial passage swine lung cells Serial passage swine lung cells were established from both normal and pneumonic lung tissue. The cell lines were adapted to growth in Madin's balanced salt solution plus LH and 10 per cent BFS without antibiotics. Serial passage of the cell lines was achieved using the technique described by L'Ecuyer (1962). The cells were transferred by replacing the growth medium with a 0.15

per cent solution of disodium dihydrogen ethylenediamine-tetraacetate dihydrate (Titraver¹) in Madin's balanced salt solution plus LH. The pH of the solution was adjusted to 7.6 with a 7.5 per cent solution of sodium bicarbonate, and the flasks were incubated at 37 C for 10 to 40 minutes. The cells became detached from the glass, and could be easily resuspended in complete growth medium to 2 or 3 times the volume of the original medium. The cell suspensions were well mixed and dispensed into flasks or tubes as required, after which they were incubated at 37 C. Cells were passaged at 5 to 10 day intervals, depending on growth rate.

Serial passage lung cells, established from lesions from experimentally produced cases of virus pneumonia of pigs, were mechanically removed from the glass surfaces, concentrated by centrifugation at 4000 G for 30 minutes, and inoculated intranasally into susceptible pigs after 3, 7 and 8 serial transfers. Pigs were necropsied 3 to 5 weeks postinoculation and examined for gross and microscopic lesions of virus pneumonia of pigs.

Cells from the 3rd serial passage were also fixed and embedded for electron microscopic examination as described under electron microscopic procedures. Cells from the 1st to the 4th serial passage grown on cover-slips in Leighton tubes were fixed and stained with Giemsa, acridine orange or orcein, prior to microscopic examination.

¹Hach Chemical Company, Ames, Iowa.

The 3rd serial passage of a swine lung cell line established from a normal pig was inoculated with a pneumonic lung suspension. Serial passage of the cell line was continued, and at the 9th passage level 3 pigs were inoculated to determine the presence of the infectious agent. After 30 days the pigs were killed and examined for gross and microscopic lesions of virus pneumonia of pigs.

Inoculation of cell cultures Cell cultures were inoculated with suspensions of pneumonic swine lung or cell culture fluids in such a way that a 10-fold dilution of inoculum resulted at each inoculation. In some instances inocula were clarified by filtration through cellulose membranes of 800 m μ APD¹ in addition to the centrifugal clarification. Daily examinations for evidence of cytopathic effect was made. Cell sheets grown on cover-slips in Leighton tubes were fixed and stained with Giemsa, acridine orange or orcein, 1 to 7 days after inoculation, and were then examined by brightfield and ultraviolet-light microscopy.

Attempts were made to demonstrate enhancement of growth of the agent of virus pneumonia of pigs in swine lung serial passage cell cultures by continued exposure to an atmosphere consisting of 95 per cent oxygen and 5 per cent carbon dioxide at a pressure of 10 pounds per square inch. The technique described by Segre (1964) was modified for use with the

¹Millipore Filter Corporation, Bedford, Massachusetts.

swine lung cell system. Normal serial passage swine lung cell cultures were grown in Madin's balanced salt solution with LH and 10 per cent bovine fetal serum as before. When the cell sheets were confluent on the 3rd or 4th day they were inoculated with a filter clarified suspension of pneumonic lung, each tube receiving 0.2 ml. inoculum. The rubber stoppers of inoculated tubes and uninoculated control tubes were replaced with sterile cotton plugs, and the tubes were taped to a sloping holder and placed in a pressure cooker modified by replacement of the control valve by a double gas cock. A pressure valve was attached to one arm of the gas cock and a mixture of 95 per cent oxygen and 5 per cent carbon dioxide was introduced via the other arm until a pressure of 10 pounds per square inch was reached. The cocks were closed and the entire container incubated at 37 C.

Cell cultures inoculated with the same inoculum, as well as uninoculated control cultures, were incubated at 37 C in the conventional way for comparison with those kept in the high oxygen environment. Every three days all cultures were transferred, and cover-slips were stained with Giemsa and with orcein for microscopic examination. After 8 serial transfers 2 groups of 3 pigs were inoculated intranasally with the infected culture grown in the high oxygen environment, and with a suspension of cells from the pooled control tubes. All pigs were killed 3 weeks later and examined for gross and

microscopic lesions of virus pneumonia of pigs. Specimens were collected for bacteriological examination.

Artificial media

Numerous attempts were made to propagate the causative agent of virus pneumonia of pigs in artificial media. A wide variety of fluid and solid media were used. Growth in fluid medium was evaluated by daily examination for turbidity, sediment or pellicle formation, and by microscopic examination of Giemsa stained sediment for evidence of microorganisms. Growth on solid medium was determined by microscopic examination using a binocular dissecting microscope and oblique lighting. The usual inoculum was a 10 per cent suspension of pneumonic lung clarified by centrifugation and sometimes by filtration through 800 $\text{m}\mu$ APD membranes. Three to 4 blind passages were usually performed at 2 to 5 day intervals with broth media, and at 5 to 15 day intervals with agar surface cultures. The latter were transferred by picking individual colonies or by cutting out 1 cm. squares of the agar containing the colonies and smearing them in the inverted position onto the surface of fresh agar plates.

Swine Mycoplasma medium The routine Mycoplasma medium used was a beef heart infusion broth containing 0.2 per cent hemoglobin, 0.5 per cent swine gastric mucin, 20 per cent turkey serum, penicillin and thallium acetate, prepared as described by Ross and Switzer (1963). Numerous attempts were

made to propagate the virus pneumonia of pigs agent in this medium and also on agar plates prepared from this medium. Agar plates were incubated on wet towels in covered stainless steel pans, or in plastic bags to maintain humidity. The agar concentrations ranged from 0.75 to 1.5 per cent agar. Modifications of the routine medium were made by substitution of the turkey serum with 5 to 15 per cent horse serum, and by the inclusion of 2.5 per cent fresh yeast extract prepared according to the procedure described by Chanock¹ which is as follows:

1. Add 250 gm. Fleischmann's Type 20 - 40 yeast² to 1 liter distilled water.
2. Heat to boiling while stirring constantly.
3. Filter through 2 sheets No. 1 filter paper.
4. Adjust pH to 8.0 by adding 0.1N NaOH.
5. Dispense in 10 to 12 ml. aliquots.
6. Autoclave at 15 pounds pressure for 15 minutes.
7. Store in freezer at -30 C.

The swine Mycoplasma broth and agar media were further modified by the inclusion of 0.1 per cent Tween 80³ prior to filter sterilization. The medium containing the Tween 80 was also

¹Dr. R. M. Chanock, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. PPL0 yeast extract. Personal communication. 1962.

²Standard Brands Incorporated, New York, N. Y.

³Hill Top Laboratories, Cincinnati, Ohio.

modified by the addition of B-diphosphopyridine nucleotide¹ (DPN) grade III at a concentration of 0.01 per cent, and L-cysteine hydrochloride² also at a concentration of 0.01 per cent as described by Chalquest (1962). One per cent solutions of DPN and L-cysteine hydrochloride were Selas filter sterilized and stored at -25 C until required.

Avian Mycoplasma media Turkey meat infusion medium containing 5 per cent yeast autolysate, penicillin and thallium acetate was prepared following the procedure described by Yoder and Hofstad (1964). Twenty per cent turkey serum or 15 per cent horse serum was added to the infusion prior to filter sterilization. Agar slants and plates, as well as broth cultures were used.

Human Mycoplasma media The agar and broth media described by Chanock³ were prepared as follows:

Mycoplasma agar:

1. Suspend 34 gm. Difco PPLO agar⁴ in 1 liter distilled water.
2. Heat and dissolve.
3. Dispense in bottles (70 ml. per bottle).

¹Sigma Chemical Company, St. Louis, Missouri.

²Nutritional Biochemicals Corporation, Cleveland, Ohio.

³Dr. R. M. Chanock, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland. PPLO broth, PPLO agar. Personal communication. 1962.

⁴Difco Laboratories, Detroit, Michigan.

4. Autoclave for 15 minutes at 15 lbs. pressure.
5. Cool at 50 C.
6. Add in the following order:
 - A. 100,000 units penicillin.
 - B. 2.5 ml. of 1:50 thallium acetate (1:2000 final dilution).
 - C. 1.0 ml. Amphotericin B¹ (0.5 mg. per ml.).
 - D. 10 ml. of 25 per cent yeast extract (as described for swine PPLO media).
 - E. 20 ml. horse serum (not inactivated).

Mycoplasma broth:

1. Dissolve 21 gm. Difco PPLO Broth² in 1 liter distilled water.
2. Dispense in 70 ml. amounts in 125 ml. bottle.
3. Autoclave for 15 minutes at 15 pounds pressure.
4. Add the following:
 - A. 100,000 units penicillin.
 - B. 2.5 ml. 1:50 thallium acetate.
 - C. 1.0 ml. Amphotericin B¹ (0.5 mg. per ml.).
 - D. 10 ml. 25 per cent yeast extract.
 - E. 20 ml. horse serum (not inactivated).

After inoculation agar plates were incubated in a humid environment.

Boiled cell medium A modification of the technique described by Goodwin and Whittlestone (1964c) was followed.

¹E. R. Squibb and Company, Franklin Park, Illinois.

²Difco Laboratories, Detroit, Michigan.

Primary pig lung cell cultures were prepared as described under "cell cultures" using Hanks' balanced salt solution with 0.5 per cent LH to which was added 0.01 per cent Difco¹ yeast extract, 20 per cent pig serum inactivated at 56 C for 30 minutes, and 200 units per ml. penicillin. On the 6th day when the cells were confluent the growth medium was poured off and the tubes were nearly filled with boiling phosphate-buffered saline and then almost totally immersed in a boiling water bath for 30 minutes followed by 10 minutes immersion after the heat had been removed. The saline was replaced with Hanks' growth medium and the tubes were then inoculated with filtered suspension of pneumonic lung and incubated at 37 C. Tubes were examined daily, and transfers to tubes containing Hanks' growth medium were made at 2 to 5 day intervals. Microscopic examination of Giemsa or orcein stained sediment from the centrifuged culture medium was performed, and results were photographically recorded with A.O.² photomicrographic equipment and Polaroid³ Type 55 P/N film.

Tissue culture medium The Hanks' growth medium as used in the boiled cell cultures was inoculated with filtered 10 per cent pneumonic lung suspension. Serial transfers and

¹Difco Laboratories, Detroit, Michigan.

²American Optical Company, Chicago, Illinois.

³Polaroid Corporation, Cambridge, Massachusetts.

microscopic examinations were performed as described above. An attempt was made to enhance the growth of the causative agent in Hanks' growth medium by inoculating the culture with Mycoplasma hyorhinis strain 7. The culture containing the two agents was scrutinized daily and serially transferred. Smears prepared from sediment after centrifugation, were examined microscopically. Medium containing no penicillin was also used to determine whether the organism would revert to a bacterial form in the absence of inhibitors.

After 7 serial passages representing a 10^{-8} dilution of the original lung inoculum, 3 groups of 3 pigs were inoculated intranasally with cultures derived from the boiled cell medium, the Hanks' growth medium and the medium containing both the pneumonia agent and M. hyorhinis. An additional 3 pigs were inoculated with the organism after 8 serial passages in Hanks' growth medium. Three control pigs were inoculated with the growth medium. All the pigs were necropsied 3 weeks postinoculation and examined for gross and microscopic lesions of virus pneumonia of pigs. Specimens were collected for bacteriological examination.

Attempts were made to grow the organisms on agar plates prepared with Hanks' balanced salt solution. One per cent Noble agar was incorporated in Hanks' balanced saline with 0.5 per cent LH, 0.01 per cent Difco¹ yeast extract and 20

¹Difco Laboratories, Inc., Detroit, Michigan.

per cent heat-inactivated swine serum. Five per cent of a suspension of normal lung which had been clarified by centrifugation at 2000 G for 15 minutes and filtration through a $650 \text{ m}\mu$ APD cellulose membrane,¹ was incorporated into some of the agar medium in an effort to enhance colony growth. The plates were poured, allowed to solidify and stored at 4 C in sealed plastic bags to prevent drying.

Agar surfaces were inoculated with pneumonic lung suspensions or with the organisms grown in artificial medium. A Micrococcus sp. nurse colony was streaked down the center of the plates which were then incubated at 37 C in a humid environment. Microscopic examinations were made at 2 to 3 day intervals to ascertain whether surface growth was occurring.

Chicken embryos

On several occasions 6 to 7 day-old embryonated hens eggs were inoculated with 10 per cent lung suspensions of virus pneumonia of pigs in an effort to propagate the agent. Prior to inoculation of eggs the suspensions were divided into 2 portions, one of which received 5000 units per ml. penicillin and 5 mg. per ml. streptomycin, while the other was not treated with antibiotics. The embryonated eggs were inoculated by the yolk-sac route, each embryo receiving 0.2 ml.

¹Millipore Filter Corporation, Bedford, Massachusetts.

inoculum. They were then incubated at 37 C. The eggs were candled daily and dead eggs were removed and kept at 4 C for at least 2 hours before examination for macroscopic lesions. On the 7th day all live embryos were chilled at 4 C prior to necropsy. Embryos were examined for gross lesions, and yolk-sac, heart, liver and spleen were aseptically collected for serial passage. Blind passages of material from both dead and live embryos were continued to the 6th passage level. Horse blood agar plates were inoculated with embryo suspensions to determine sterility.

Yolk-sac smears were prepared, stained with Giemsa's stain or by Macchiavello's technique, and examined microscopically.

Seven day-old embryonated eggs were inoculated with 0.2 ml. of Hanks' medium which had been inoculated with virus pneumonia of pigs agent 4 days previously. After 2 serial passages in eggs a yolk-sac and embryo suspension was prepared, mixed with a suspension prepared from 6th serial passage eggs, and inoculated intranasally into 3 experimental pigs. The inoculations were repeated twice at 2 day intervals. The pigs were killed 20 days after initial exposure, and examined for gross and microscopic lesions of virus pneumonia of pigs. Specimens were collected for bacteriological examination.

Ten day-old embryonated hens eggs were inoculated onto the chorio-allantoic membrane with a 800 μ APD

filter-clarified lung suspension of virus pneumonia of pigs to determine whether pock or plaque formation could be demonstrated. The eggs were candled daily and examined for lesions after seven days incubation.

Mice

Several attempts were made to infect mice with the causative agent of virus pneumonia of pigs. Six 21 day-old Carworth Farm (CFW) mice were inoculated intranasally with a 10 per cent suspension of pneumonic lung. They were killed after 21 days and lungs were collected for serial passage and for histological examination. Mice inoculated with 2nd serial passage lung suspension were necropsied after 14 days and examined for pneumonia or other lesions.

On three occasions baby mice between 1 and 5 days old were inoculated intranasally with lung suspensions of virus pneumonia of pigs. They were necropsied after 2 to 5 weeks, and lungs were collected for histological examination and for serial passage. Stored passage material from each of these groups of mice was pooled and passaged serially at 14 day intervals in baby mice for 2 serial passages. Sections of lungs were collected for histological examination after each passage.

Histological Procedures

Tissue sections

Tissues were collected and immersed in fixative within 5 minutes after death of the experimental pigs. Ten per cent buffered formalin or Bouin's fixative plus 5 per cent acetic acid was used, and in all cases a minimum fixation time of 48 hours was allowed. Tissues were removed, trimmed and processed immediately, or stored in 70 per cent ethyl alcohol. Tissues were embedded in Paraplast¹ tissue embedding medium, sectioned at 6 μ and mounted on glass slides with an albumin fixative. Sections were stained with Giemsa or with Harris' hematoxylin and counterstained with eosin Y as described in the U. S. Armed Forces Institute of Pathology (1960) Manual of Histologic and Special Staining Technics.

Cell culture coverslips

Cells grown on coverslips in Leighton tubes were examined following staining with Giemsa, acridine orange or orcein stain.

Giemsa staining Coverslips were removed from Leighton tubes, fixed in Bouin's fixative plus 5 per cent acetic acid, dehydrated and stained with 2 per cent Giemsa stain for 4 to 24 hours as described by Switzer (1959).

¹Aloe Scientific, St. Louis, Missouri.

Acridine orange staining The technique used was a modification of the method described by Pollard and Tanami (1962). After removal from the Leighton tubes the coverslips were stained as follows:

1. Fix in 3 per cent HCl in 95 per cent ethanol. 5 minutes
2. Rinse in 2 changes of McIlvaine's buffer, pH 3.6. 2 minutes
3. Stain with .01 per cent acridine orange in McIlvaine's buffer. 4 minutes
4. Rinse in 2 changes of McIlvaine's buffer. 2 minutes
5. Mount coverslip in McIlvaine's buffer and seal with melted paraffin.

McIlvaine's buffer solution was prepared from the following stock solutions of citric acid and disodium phosphate;

Stock solution A 0.01M citric acid

Stock solution B 0.02M Na_2HPO_4

A pH of 3.6 was achieved by mixing the 2 solutions in the following proportions:

Citric acid 13.56 ml.

Disodium phosphate 6.44 ml.

Acridine orange stained preparations were examined immediately after staining with an AO microscope¹ equipped with a darkfield condenser and photographic accessories. An AO "Fluorolume" illuminator with an Osram HBO 200 mercury vapor

¹American Optical Company, Chicago, Illinois.

arc lamp served as the ultraviolet light source. A 3 mm. Schott BG-12 exciter filter, and a Schott OG-1 barrier filter were used in the system.

Orcein staining A modification of the procedure described by Fogh and Fogh (1964) was used to stain normal and virus pneumonia of pigs infected cells grown on coverslips in Leighton tubes. This was done as follows:

1. Replace culture medium with 1.5 ml. of 0.6 per cent sodium citrate solution.
2. Add 0.5 ml. distilled water drop by drop (final concentration of sodium citrate is 0.45 per cent).
3. Allow to stand for 10 minutes.
4. Slowly add an equal amount of Carnoy's fixative.
5. Replace this mixture with Carnoy's fixative.
6. Fix for 10 minutes.
7. Remove slide from tube and allow to dry for at least 5 minutes.
8. Stain for 5 minutes with orcein stain.
9. Wash 3 times in absolute alcohol.
10. Mount in Euparal¹.

The Carnoy's fixative was prepared as follows:

glacial acetic acid	1 part
absolute ethyl alcohol	3 parts

¹Flatters and Garnett Ltd., Manchester, England.

The 2 per cent solution of synthetic orcein¹ was prepared by dissolving the orcein in boiling glacial acetic acid, cooling to 55 C and then adding distilled water to give a final acetic acid concentration of 60 per cent. This was cooled to room temperature and filtered twice through Whatman No. 1 filter paper.

Touch preparations Touch preparations were prepared by gently pressing clean microscope slides against freshly cut surfaces of virus pneumonia of pigs lung lesions. The impressions were then air-dried, fixed in methyl alcohol for 3 minutes and stained for 90 to 120 minutes in 2 per cent Giemsa solution in distilled water. Slides were washed and air-dried prior to microscopic examination.

Broth cultures Culture media suspected of containing growing organisms were centrifuged at 4000 G for 15 minutes, the supernatant fluid was aspirated off, and smears were made from the button in the centrifuge tube. The smears were air-dried, fixed in absolute methyl alcohol and stained with Giemsa as described for touch preparations. Smears were also stained by Gram's method prior to microscopic examination.

¹Matheson, Coleman and Bell, Cincinnati, Ohio.

Electron Microscopy Procedures

Collection and fixation of specimens

Lung specimens were collected from normal or experimentally infected virus pneumonia pigs while under deep barbiturate anesthesia. Normal lung specimens were also collected immediately after killing pigs for cell culture preparation. The tissues were cut into very small pieces and fixed in osmium tetroxide at 4 C or in glutaraldehyde. Specimens collected in osmium tetroxide fixatives were held for 1 to 2 hours at room temperature before commencing dehydration. Tissues fixed in Kellenberger's fixative were held at 4 C overnight before dehydration. Tissues fixed in glutaraldehyde were held for 1 to 24 hours, or if longer storage was required, they were washed in acetate-veronal buffer after 1 hour and stored in 0.2 M sucrose for up to 1 month. All glutaraldehyde fixed tissues were postfixed in osmium tetroxide fixatives.

Cell cultures grown from normal or virus pneumonia lungs were usually fixed in osmium tetroxide containing sucrose and CaCl_2 , but on occasion the CaCl_2 was not included. Cells were either fixed in situ or they were loosened from the glass with a sterile latex tube and then fixed.

Buffer solution

The acetate-veronal buffer stock solution described by Palade (1952) was prepared as follows:

Na-veronal (sodium barbital)	14.714 gm.
Na-acetate, crystalline ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$)	9.714 gm.
Water	500 ml.

Fixatives

One per cent osmium tetroxide solution (Palade, 1952) was prepared as follows:

2 per cent stock solution OsO_4	12.5 ml.
Acetate-veronal buffer	5.0 ml.
0.1N HCl	4 to 5 ml. to give pH 7.4

Make up to 25 ml. with distilled water.

The OsO_4 -sucrose fixative described by Caulfield (1957) was used to reduce cellular distension by raising the osmotic pressure. The fixative was prepared as follows:

Prepare 1 per cent OsO_4 as above.

Add 0.045 gm./ml. sucrose.

This fixative was modified by the addition of 1.0 ml. of 0.04 M CaCl_2 .

The fixative described by Kellenberger et al. (1958) as being optimal for the preservation of fine structure of bacteria was prepared as follows:

Michaelis buffer.

Na-acetate	19.428 gm.
Na-veronal	29.428 gm.
NaCl	34.000 gm.
Distilled water	1000 ml.

Using this buffer the following fixative was prepared:

Buffer	5.0 ml.
0.1N HCl	7.0 ml.
Distilled water	13.0 ml.
M-CaCl ₂	0.25 ml.
OsO ₄	0.25 gm.

The final pH of this solution was 6.0.

The 2.5 per cent glutaraldehyde fixative described by Sabatini et al. (1963) was used when processing of tissues could not commence within 1 hour after collection. The fixative was prepared as follows:

25 per cent glutaraldehyde	1.0 ml.
Acetate-veronal buffer	2.0 ml.
Distilled water	6.0 ml.

Adjust to pH 7.4 with 0.1N HCl.

Make up to 10 ml. with distilled water.

Embedding media

A 9:1 n-butyl:methyl methacrylate monomer solution was prepared as follows:

n-Butyl methacrylate	90 ml.
Methyl methacrylate	10 ml.
Benzoyl peroxide	2 gm.

A modification of the methacrylate-divinyl benzene embedding medium described by Kushida (1961) was prepared for use as follows:

n-Butyl methacrylate	98.5 ml.
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Divinyl benzene 55 ¹	1.5 ml.
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Benzoyl peroxide	1.0 gm.
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The epon embedding medium described by Luft (1961) was prepared in the following way:

A. Epon 812	62 ml.
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Dodecenyl succinic anhydride (DDSA)	100 ml.
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Mix well.

B. Epon 812	100 ml.
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Methyl nadic anhydride (MNA)	89 ml.
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Mix well.

Equal quantities of stock solutions A and B were mixed when required for embedding, and 1.5 per cent of the accelerator 2, 4, 6 - dimethylamino-methyl-phenol (DMP-30) was added.

Dehydration and infiltration

Dehydration and methacrylate infiltration of tissues was achieved as follows:

25 per cent ethanol	15 minutes
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50 per cent ethanol	15 minutes
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70 per cent ethanol	15 minutes
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85 per cent ethanol	15 minutes
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95 per cent ethanol	15 minutes
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absolute ethanol (2 changes)	8 minutes each
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50:50 absolute ethanol:methacrylate	30 minutes
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¹Dow Chemical Company, Midland, Michigan.

Methacrylate plus Benzoyl peroxide 30 minutes

Pre-polymerized methacrylate 18 hours

The schedule for dehydration and infiltration of tissues with epon was as follows:

25 per cent ethanol 15 minutes

50 per cent ethanol 15 minutes

70 per cent ethanol 15 minutes

95 per cent ethanol 15 minutes

absolute alcohol (2 changes) 10 minutes each

propylene oxide (2 changes) 15 minutes each

Immersion in a 50:50 propylene oxide:epon mixture for 1 to 4 hours was followed by deposition of the tissue in gelatin capsules containing epon.

The tissues were occasionally held in 70 per cent ethanol overnight.

Polymerization

The 9:1 n-butyl-methyl metnacrylate was polymerized in an oven at 55 C, or by exposure to ultraviolet light (Westinghouse Sun Lamp¹) at a distance of 2 cm. cooled by air-flow from an exhaust fan. Methacrylate containing divinyl benzene was polymerized by neat only. Epon was polymerized over a period of 2 days as follows:

35 C	18 hours
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¹Westinghouse Appliance Sales, Des Moines, Iowa

45 C	8 hours
60 C	18 hours

Tissue fragments such as lung specimens were dehydrated and infiltrated in glass tubes and embedded in gelatin capsules. Cells from cultures grown on glass were processed in a number of ways. The technique described by Howatson and Almeida (1958) was used for the fixation, dehydration, infiltration and embedding of cells while still adhering to glass coverslips. The fixation, dehydration and infiltration procedures were as described previously. Prior to polymerization a gelatin capsule partly filled with viscid pre-polymerized methacrylate was inverted on the cells, and heat or ultra-violet light polymerization was then continued. When polymerization was complete, the coverslips with attached capsules were placed on solid CO₂ for 2 minutes, after which the capsules with the embedded cells could be snapped off. Cells which were loosened from the glass surfaces prior to or after fixation were dehydrated and infiltrated in centrifuge tubes, each 15 minute dehydration step being performed in a centrifuge at 2000 G. Gelatin capsules containing cell suspensions were centrifuged at 2000 G for 15 minutes to settle the suspended cells prior to polymerization.

Sectioning

Capsules were suitably trimmed and sections were cut using a Cambridge Huxley¹ ultramicrotome and an LKB² ultramicrotome. Glass knives were used for all methacrylate sections and for most of the epon sections. A diamond knife was used for the remainder. Sections were cut between 50 and 80 m μ thickness. Methacrylate sections were flattened with chloroform or xylene vapors, and floated onto 200 mesh grids coated with a thin layer of parlodion. Epon and methacrylate-divinyl benzene sections were placed on 300 mesh grids with no support films.

Staining

Sections were stained with lead citrate as described by Reynolds (1963) for 10 to 20 minutes, or with 1 per cent uranyl acetate (Watson, 1958) for 1 to 2 hours. Stained methacrylate sections were overlaid with a thin film of methacrylate to reduce sublimation in the beam (Roth, 1961). This procedure was not followed for epon or methacrylate-divinyl benzene embedded specimens.

Electron microscopes

The early part of this work was performed using an RCA

¹Cambridge Instrument Company Limited, London, England.

²L.K.B. Produkter A.B., Stockholm, Sweden.

EMU 3F¹ electron microscope operated at 50 or 100 kv, with a 200 μ condenser aperture and a 30 μ objective aperture. A Hitachi HU 11A² electron microscope operated at 50 kv with a 300 μ condenser aperture and a 50 μ objective aperture was used for the latter part of the work.

Filtration Studies

The particle size of the virus pneumonia of pigs agent was determined by filtration through a graded series of Millipore³ cellulose membrane filters supported in Millipore³ Micro-Syringe filter holders and mounted as illustrated in Figure 1. Fiberglass clarifying pads were used in conjunction with the 800 μ APD filters only. Positive pressures not exceeding 10 psi were used to force the suspensions through the filters.

In the first trial a suspension of infective lung was prepared and clarified by centrifugation at 2000 G for 10 minutes and filtration through a membrane of 800 μ APD. This clarified suspension was then divided into four aliquots; one served as control inoculum and the other three were passed through membranes of 650 μ , 450 μ and 300 μ APD respectively.

¹Radio Corporation of America, Camden, New Jersey.

²Hitachi, Limited, Tokyo, Japan.

³Millipore Filter Corporation, Bedford, Massachusetts.

Figure 1. Filtration apparatus with air-pressure hose attached.



Sixteen pigs were divided into 4 groups of 4 and housed separately. Each group was inoculated intranasally with 1 ml. of one of the above-mentioned filtrates. These inoculations were repeated 48 hours later with filtrates which had been stored at -30 C in the interim. The pigs were killed 30 days postinoculation, examined for gross lesions, and specimens were collected for bacteriological and histological evaluation.

A "marker" virus of known size was included in the second filtration trial as a check on the integrity of the filter membranes. A strain of fowl-pox virus was obtained through the courtesy of Dr. M. S. Hofstad¹, and a preliminary size determination of this agent was performed to determine its suitability as a "marker" virus. Virus suspensions were filtered through membranes of 800 m μ , 450 m μ , 300 m μ and 220 m μ APD, and assayed for virus by inoculation of the chorio-allantoic membranes of 10 day-old embryonated hens eggs. The virus passed the 300 m μ but not the 220 m μ APD membranes. Fowl-pox virus was mixed with a suspension of pneumonic lung to give a final dilution of 10^{-2} of the stock fowl-pox virus. The mixture was clarified through an 800 m μ APD membrane, and then divided into four aliquots, three of which were filtered through membranes with APD of 450 m μ , 300 m μ and 220 m μ respectively. Chorio-allantoic membrane inoculations of 10 day-old eggs were made with each of the filtrates, and also

¹Dr. M. S. Hofstad, Iowa Veterinary Medical Research Institute, Ames, Iowa.

with fowl-pox virus and virus pneumonia of pigs lung suspension.

Sixteen pigs were divided into 4 groups of 3 pigs and 1 group of 4 pigs. Each group of pigs was inoculated intranasally with one of the following inocula; fowl-pox virus or 800 m μ , 450 m μ , 300 m μ and 220 m μ membrane filtrates of the mixed suspension. The pig inoculations were repeated on the 3rd and 5th days after the initial exposure. All pigs were killed 21 days after the first inoculation, and examined for gross lesions of pneumonia and for pox lesions. Specimens were collected for bacteriological, histological and virus evaluation.

Serological Procedures

Preparation of antisera

Four 3 month-old lambs were bled for serum 5 weeks prior to inoculation and again 3 days preinoculation. Two lambs were inoculated intramuscularly with 5 ml. of a 1:1 mixture of 10 per cent suspension of pneumonic lung and 4 per cent sodium alginate adjuvant (Algivant¹). The injections were repeated on the 3rd, 5th, 16th, and 63rd days. The lung used for preparation of the inoculum was shown to be infective by pig inoculation. The other 2 lambs were injected

¹Colab Laboratories Incorporated, Chicago Heights, Illinois.

intravenously with 1.5 ml. of the 10 per cent lung suspension. The injections were repeated on the same days as the intramuscular ones. All 4 lambs were bled for postinoculation sera 1, 2, 3, 4, and 9 weeks after the first inoculation. They were bled again 10 days after the booster inoculation which was given on the 63rd day.

One 6 month-old boar from the respiratory disease free herd was bled for serum prior to inoculation with a 20 per cent suspension of pneumonic lung. Two ml. of the lung suspension was injected intravenously, and 2 ml. of a 1:1 mixture of the lung suspension and Difco¹ Complete Freunds Adjuvant was injected intramuscularly and subcutaneously. The intravenous inoculation was repeated on the 2nd day, and the intramuscular and subcutaneous inoculations on the 2nd, 4th, 15th, and 30th days postinoculation. Serum was collected on the 30th day and again on the 40th day which was 10 days after the booster inoculation.

Agar-gel diffusion micro-precipitation tests

The double-diffusion technique of Ouchterlony (1949) and the single-diffusion method of Ouidin (1948) were used in efforts to demonstrate antibodies to the agent of virus pneumonia of pigs in swine and sheep antisera.

The diffusion agar was prepared as follows:

¹Difco Laboratories, Detroit, Michigan.

0.85 per cent NaCl	45 ml.
0.15M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	35 ml.
0.15M KH_2PO_4	15 ml.
Aqueous merthiolate	5 ml.
Noble agar ¹	1 gm.

Autoclave at 15 pounds pressure for 15 minutes,

The pH of this mixture was 7.2.

For the Ouchterlony double-diffusion tests 8 ml. hot agar was pipetted into 50 mm. Petri dishes and allowed to congeal at room temperature. For the Ouidin single-diffusion test agar was placed in a water-bath at 56 C and the temperature was allowed to stabilize before addition of sera.

Four-week postinoculation serum from each of 4 sheep was adsorbed with acetone-extracted lung powder prepared from normal pig lung and adjusted to pH 7.2 in phosphate buffered saline. The adsorption was performed as follows:

postinoculation serum	4 ml.
lung powder	50 mgm.

Shake well and stand overnight at 4 C.

Sediment powder at 12,100 G for 15 minutes and remove serum.

Heat serum at 56 C for 30 minutes.

In the Ouidin test, preinoculation serum, 4-week post-inoculation serum and adsorbed 4-week postinoculation serum

¹Difco Laboratories, Detroit, Michigan.

from each of 4 sheep was incorporated into the agar at the rate of 5 ml. serum to 35 ml. agar, pipetted into 10x1 cm. test tubes, and allowed to congeal. Antigens consisting of finely ground normal or pneumonic lungs which had been quick-frozen and thawed 4 times were layered on the surface of the serum-containing agar in the tubes. Duplicate sets were kept at room temperature (26 C) and at 37 C and observed daily for 10 days for lines of precipitation.

In performing the Ouchterlony tests, both 1 per cent and 1.5 per cent agar were used. Six wells 6 mm. in diameter were cut concentrically around an identical central well at a distance of 6 mm. Preinoculation sera, 4-week postinoculation sera and adsorbed 4-week postinoculation sera were pipetted into the central wells of plates prepared in this way. The peripheral wells were then filled with the following:

1. phosphate-buffered saline (PBS)
2. concentrated suspension of pneumonic lung in PBS
3. concentrated suspension of normal lung in PBS
4. concentrated suspension of feline pneumonitis infected yolk-sac in PBS
5. Bordetella bronchiseptica infected yolk
6. concentrated suspension of pneumonic lung in PBS

All the antigens were quick-frozen and thawed 4 times before use. Plates were kept at room temperature (26 C) and at 37 C

and observed daily for 12 days for evidence of precipitation lines.

Skin sensitivity tests

An attempt was made to develop a skin sensitivity test for use in the diagnosis of virus pneumonia of pigs. Two techniques were used for the preparation of antigens for the skin tests. A modification of the technique described by Gourlay (1964) for the preparation of skin-test antigen for Contagious Bovine Pleuropneumonia was used as follows:

1. Prepare 20 per cent suspensions of normal and pneumonic lung in phosphate-buffered saline pH 7.2.
2. Centrifuge at 1500 G for 15 minutes and collect 10 ml. supernatant fluid.
3. Add an equal volume of 5 M urea, giving a final concentration of 2.5 M urea.
4. Disintegrate antigen particles at 4 C for 1 hour using a Branson LS-75 Sonifier¹ at 30 kilo-cycles/sec. at maximum power.
5. Add equal volume of $(\text{NH}_4)_2\text{SO}_4$ saturated at 4 C. Mix well and stand overnight at 4 C.
6. Centrifuge at 2000 G for 30 minutes at 4 C.
7. Remove supernatant fluid, resuspend sediment in 12 ml. 2.5 M urea, add equal amount of $(\text{NH}_4)_2\text{SO}_4$ as before, mix and stand overnight at 4 C.
8. Centrifuge at 2000 G for 30 minutes at 4 C.

¹Branson Ultrasonic Corporation, Stamford, Connecticut.

9. Resuspend sediment in 5 ml. 2.5 M urea.
10. Store at 4 C in sealed vials until required.

The second technique used for antigen preparation was a modification of the method described by Barwell (1952) for the preparation of Psittacosis antigens. The antigen was prepared as follows:

1. Prepare 20 per cent suspensions of normal and pneumonic lung in phosphate-buffered saline pH 7.2.
2. Centrifuge at 1500 G for 15 minutes and collect 5 ml. supernatant fluid.
3. Immerse in boiling water bath for 20 minutes.
4. Seal in vials and store at 4 C until required.

Eight pigs which had been inoculated intranasally with pneumonic lung 6 weeks previously were used in the trial. Each pig was inoculated intradermally with 0.1 ml. of each of the control and pneumonia antigens, injections being made in the thin skin in the anterior sternal region. Skin measurements were made prior to inoculation and again after 24, 48 and 72 hours. Temperatures were taken each time prior to measuring the skin. The pigs were necropsied at the termination of the trial, and all 8 were found to have gross and microscopic lesions of virus pneumonia of pigs.

On the basis of a thermal response observed in the first trial, a second trial was performed in which 3 virus pneumonia infected pigs and 3 normal pigs were each inoculated intradermally with 0.1 ml. and subcutaneously with 0.5 ml. of the

pneumonia antigens. Temperatures were taken prior to inoculation and after 24 and 48 hours. The pigs were necropsied at the termination of the trial, and gross and microscopic lesions of virus pneumonia of pigs were found in all 3 of the infected pigs, but no lesions were present in the uninoculated controls.

Complement fixation test

An attempt was made to demonstrate complement fixing antibodies to the Psittacosis group of agents in the sera of sheep inoculated with the causative agent of virus pneumonia of pigs. The tests were performed through the courtesy of Dr. L. A. Page¹ who also supplied the antigens. The direct complement fixation technique described by Page and Bankowski (1960) was used. The serum titers were expressed as the reciprocal of the highest dilution of serum giving complete fixation of 2 exact units of complement with 2 units of antigen. All serum dilutions were made after inactivation of serum at 56 C for 30 minutes.

The test was set up by mixing 0.25 ml. of the appropriate serum dilution with 0.25 ml. complement, 0.25 ml. antigen, and 0.25 ml. veronal buffered saline, pH 7.2. The mixtures were incubated at 37 C for 120 minutes, after which 0.5 ml. sensitized sheep erythrocytes were added to each tube. After

¹Dr. L. A. Page, National Animal Disease Laboratory, Ames, Iowa.

shaking thoroughly the mixtures were held at 4 C overnight. The tests were read the following morning, the titer being the highest dilution that completely resisted hemolysis.

Sheep preinoculation sera as well as 1, 2, 3, 4, 9 and 10 week postinoculation sera were tested for antibodies against 4 different psittacosis-group antigens and normal chicken yolk sac antigen. The 4 psittacosis antigens were:

1. Turkey ornithosis grown in chicken yolk-sac.
2. Turkey ornithosis grown in duck yolk-sac.
3. Pigeon ornithosis grown in chicken yolk-sac.
4. Pigeon ornithosis grown in duck yolk-sac.

The appropriate cell, hemolysin and serum controls were included.

Fluorescent antibody techniques

An attempt was made to develop an indirect fluorescent antibody technique for use in the diagnosis of virus pneumonia of pigs. The antisera used in the tests were from sheep and a boar prepared as described previously.

Antigen preparation The organism which was isolated from pneumonic lung was propagated in Hanks' balanced salt solution plus 0.5 per cent LH, 0.01 per cent Difco¹ yeast extract, and 20 per cent heat-inactivated "Hypogamma Calf

¹Difco Laboratories, Inc., Detroit, Michigan.

Serum¹". After 7 days incubation at 37 C a distinct spiral was observed in tubes that were shaken gently, and it was assumed that growth had occurred. The organisms were sedimented by centrifugation at 4000 G for 20 minutes, and the button was resuspended in a few drops of the growth medium. One drop of the suspension was placed onto each of several clean slides, air-dried and fixed by gentle heating over a flame. One smear was fixed with methyl alcohol, stained with Giemsa, and examined microscopically to confirm the presence of the organisms. Mycoplasma granularum was propagated in the same medium and smears were prepared as described for the agent of virus pneumonia of pigs.

Staining procedure Two to 3 drops of preinoculation and postinoculation boar serum were applied to separate slides which were then placed in a humidity chamber to prevent evaporation, and incubated at 37 C for 30 minutes. The serum was washed off with pH 7.2 Bacto-FA buffer, and a drop of Bacto-FA-Porcine Globulin Antiglobulin (Rabbit) was applied to the surface of each slide. In addition the conjugate was applied to slides which had not been treated with preinoculation or postinoculation serum. The slides were placed in a humidity chamber and held at room temperature for 30 minutes. They were then washed in the buffer for 10 minutes with 2 changes of the buffer solution. The slides were blotted dry

¹Grand Island Biological Company, Inc., Grand Island, N.Y.

between 2 pieces of lint-free absorbent paper, and cover-slips were applied using Bacto-FA Mounting Fluid¹. The tests with sheep sera were performed in the same way except that Bacto-FA-Sheep Globulin Antiglobulin (Rabbit)¹ conjugate was used.

Microscope The slides were examined with an AO² microscope equipped with a Fluorolume² illuminator and the appropriate filters as described previously for acridine orange staining.

Drug Sensitivity

Tylosin sensitivity

Three experiments have been performed to determine the in vivo sensitivity of the agent of virus pneumonia of pigs to Tylosin tartrate³ (Tylan Injectable). The first experiment was designed to demonstrate the prophylactic effect of a single intramuscular injection of Tylosin tartrate on the development of lesions of virus pneumonia of pigs. An additional objective was to determine the effect of uncomplicated virus pneumonia of pigs on the growth rate of the affected pigs.

¹Difco Laboratories, Inc., Detroit, Michigan.

²American Optical Company, Chicago, Illinois.

³Eli Lilly and Company, Greenfield, Indiana.

Twenty-four pigs approximately 6 weeks old were divided into 3 groups of 8 by random numbers selection and housed in separate isolation units. All the pigs were weighed at the start of the experiment, again after 4 weeks and at the termination of the experiment after 10 weeks. Daily approximations of feed and water consumption were made throughout the experiment.

Two of the 3 groups were inoculated intranasally with a 10 per cent suspension of virus pneumonia of pigs lung suspension. The inoculations were repeated on the 1st, 3rd and 4th days after initial exposure. One group of pigs received a single deep intramuscular injection of 10 mg./kilogram of Tylosin tartrate on the 10th day after initial inoculation with the agent of virus pneumonia of pigs. All the pigs were killed between 10 and 11 weeks postinoculation and examined for gross lesions. The location and extent of lesions were recorded and specimens were collected for histological and bacteriological examination. Primary swine kidney cell cultures were inoculated with pneumonic lung suspension and examined daily in an effort to demonstrate cytopathogenic viruses.

The effect of long-term oral administration of Tylosin tartrate¹ (Tylan 40) on established cases of virus pneumonia of pigs was evaluated. Twelve pigs were inoculated

¹Eli Lilly and Company, Greenfield, Indiana.

intranasally with a 10 per cent virus pneumonia of pigs lung suspension. The inoculations were repeated 3 times at 2 day intervals, and the pigs were observed until the 21st day after initial inoculation. By a process of random selection the pigs were divided into 2 groups and housed separately. One group was fed a complete ration containing 1000 gm./ton Tylosin tartrate while the other group received the same basal ration without medication. Seven weeks later all the pigs were killed and examined for gross pneumonic lesions. Specimens were collected for histological and bacteriological examination, and also for pig inoculation to determine whether the agent was still present in the lesions. Three pigs were inoculated twice at 2 day intervals with a suspension prepared from pooled lungs collected from the treated group. These pigs were killed 8 weeks later and examined for gross and microscopic evidence of virus pneumonia of pigs.

An experiment was performed to determine whether pigs receiving feed containing high levels of Tylosin tartrate (Tylan 40) at the time of inoculation with the agent of virus pneumonia of pigs, would be resistant to infection. Eight pigs were divided into 2 groups of 4 and housed separately in isolation. The untreated control group were fed a complete basal ration with no drug additives throughout the trial, while the treated group were fed the same ration to which had been added 1000 gm./ton Tylosin tartrate.

The pigs were fed these feeds for 7 days before all the pigs in both groups were inoculated intranasally with a 10 per cent pneumonic lung suspension. The inoculations were repeated twice at 2 day intervals. All the pigs were killed 21 days after initial exposure to the agent and examined for gross and microscopic lesions of virus pneumonia of pigs. Bacteriological examinations were made of specimens collected from the lungs. Touch preparations were made from freshly cut lesions, stained with Giemsa and orcein and examined microscopically.

A trial was performed to determine the in vitro sensitivity to Tylosin of the organism isolated in artificial medium from pneumonic lung. A series of dilutions of Tylosin tartrate¹ ranging from 100 $\mu\text{gm.}/\text{ml.}$ to 0.05 $\mu\text{gm.}/\text{ml.}$ were made in the Hanks' growth medium. The tubes containing the drug dilutions were inoculated with 4th serial passage culture of the organism each tube receiving the same inoculum. One control tube was inoculated with the organism and no drug and another received 100 $\mu\text{gm.}/\text{ml.}$ drug but no organisms. All tubes were examined for evidence of growth on the 2nd, 3rd and 4th days postinoculation.

¹Eli Lilly and Company, Greenfield, Indiana.

Furaltadone sensitivity

An experiment was performed to determine the in vivo sensitivity of the agent of virus pneumonia of pigs to Furaltadone¹ (Valsyn Water Mix). Thirty-four pigs approximately 6 weeks old were divided by random selection into 4 groups, 2 with 9 pigs and 2 with 8 pigs. One group of 9 pigs was started on Valsyn Water Mix at a level of 6.6 gm./gal. of drinking water 2 days prior to inoculation, and continued on this medication for 21 days. Waterers were drained, cleaned and replenished with medicated water every 3 or 4 days. A 2nd group of 9 pigs was started on the same regimen of Valsyn treatment on the 13th day after intranasal inoculation with pneumonic lung suspension. The treatment was continued for 21 days, discontinued for 18 days, and then renewed and continued for 24 days at which time the 2 treated groups together with the uninoculated control group and the inoculated untreated control group were killed. The pigs were examined for gross lesions and specimens were collected for histological, bacteriological and virological examination.

Chlortetracycline sensitivity

An experiment was performed to determine the in vivo sensitivity of the agent of virus pneumonia of pigs to chlortetracycline. Nine pigs were divided into 2 groups, one

¹Norwich Pharmacal Company, Norwich, New York.

of 4 and one of 5 pigs. The smaller group was fed a basal ration containing no drug. The other group received the same ration to which had been added 400 gm./ton chlortetra-cycline in the form of Eurofac 10¹. One week after the pigs were started on these feeds, they were all inoculated with a 10 per cent lung suspension of virus pneumonia of pigs. The inoculation was repeated twice at 2 day intervals. All the pigs were killed 21 days after the first inoculation and examined for gross pneumonic lesions. Specimens were collected for histological and bacteriological examination, and touch preparations were made from freshly-cut lung.

Ether Sensitivity

The ether sensitivity of the virus pneumonia of pigs agent was determined using the technique described by Andrewes and Horstmann (1949). A 10 per cent suspension of infected swine lung suspended in Dulbecco's phosphate broth (DPB) plus calf serum was centrifuged at 2000 G for 10 minutes. Eight ml. of the supernatant fluid was placed in each of 2 screw-capped tubes. Two ml. of anhydrous ether² (ether absolute) was added to one tube to give a final concentration of 20 per cent ether by volume. Two ml. of DPB plus calf serum was added to the other tube to bring its volume to 10 ml. Both

¹American Cyanamid Company, Princeton, New Jersey.

²Mallinckrodt Chemical Works, New York, N.Y.

tubes were vigorously shaken and placed in a refrigerator at 4 C for 24 hours. The contents of each tube was then poured into a sterile petri dish with the cover partly open and left for 30 minutes to allow the ether to evaporate. Both preparations were treated similarly.

Eight pigs were divided into two groups of 4 and housed separately. One group was inoculated intranasally with the ether-treated suspension while the control group received the untreated suspension. Each pig received 2 ml. of the inoculum. The pigs were necropsied 8 weeks postinoculation and representative samples were collected for histological and bacteriological examination.

Viral Interference

An experiment was performed to determine whether the virus pneumonia of pigs agent inoculated into cell cultures would interfere with the growth of six different viruses. Primary swine kidney cell cultures, prepared according to a previously reported technique (Switzer, 1959), were inoculated with a 10 per cent suspension of virus pneumonia of pigs lung which had been filter-clarified through an 800 m μ APD membrane. The inoculated cell cultures were incubated for 48 hours at 37 C and used to titrate the following 6 viruses:

1. Newcastle disease virus (NDV), G.B. strain which had been adapted to growth in primary swine kidney cell cultures and passaged 72 times. An easily recognizable cytopathic effect was present in cell cultures 2 days postinoculation.

2. Pseudorabies virus isolated in serial passage swine kidney cell cultures from infected rabbit brain kindly supplied by Dr. R. E. Shope¹. This virus was further passaged once in primary swine lung cell cultures and 3 times in primary swine kidney cell cultures.
3. Virus BTS-43 isolated in primary swine kidney cell cultures from the nasal cavity of a pig, and passaged 15 times in primary swine kidney cell cultures.
4. Ohio ECPO-1 virus kindly supplied by Dr. E. H. Bohl², and passaged 5 times in primary swine kidney cell cultures since receipt.
5. Sturdy virus isolated from a rectal swab from a pig with diarrhea, and passaged 32 times in primary swine kidney cell cultures.
6. Infectious Canine Hepatitis (ICH) virus.³

The Sturdy, BTS-43 and Newcastle disease viruses were supplied by Dr. W. P. Switzer⁴.

Control titrations were run in cells which had not been inoculated with the virus pneumonia of pigs agent.

The BTS-43 and Ohio ECPO-1 viruses produced the type 2 cytopathic effect described by Switzer and L'Ecuyer (1960). This change is characterized by incomplete rounding and granularity of affected cells, followed by nuclear and cytoplasmic

¹Dr. R. E. Shope, Rockefeller Institute, New York, N.Y.

²Dr. E. H. Bohl, Department of Bacteriology, Ohio State University, Columbus, Ohio.

³Infectious Canine Hepatitis Vaccine, Modified Live Virus, Fromm Laboratories, Grafton, Wisconsin.

⁴W. P. Switzer, D.V.M., Iowa Veterinary Medical Research Institute, Ames, Iowa.

disintegration and formation of cytoplasmic strands. Switzer¹ has shown that they are serologically distinct. The Sturdy virus produced the type 3 cell destruction characterized by rounding and enlargement of affected cells followed by disintegration, clumping and release of the cells from the glass. According to Switzer¹, this swine enterovirus is serologically distinct from the previous two.

Virus titrations were performed by serial 10-fold dilution of the viruses from 10^{-0} to 10^{-7} , followed by inoculation of cell culture tubes containing 2 ml. culture medium. Each tube received 0.2 ml. of the virus dilution. This resulted in final virus dilutions of 10^{-1} to 10^{-8} . The infected cell sheets were examined daily for evidence of cytopathic effects, and the results were recorded. Fifty per cent end points were calculated by the method of Reed and Muench (1938).

The virus pneumonia of pigs inoculum used to infect the cell sheets was subsequently shown to be viable by pig inoculation.

Experimental Mycoplasma pneumoniae Infection of Swine

The strain of Mycoplasma pneumoniae (Eaton agent) used for swine inoculation was obtained through the courtesy of

¹Dr. W. P. Switzer, Iowa Veterinary Medical Research Institute, Ames, Iowa. Serological relationships between swine enteroviruses. Personal communication. 1965.

Dr. R. M. Chanock¹. A 6 day-old culture of the agent grown in the broth medium described by Chanock et al. (1962b) served as the inoculum. Each of 2 pigs received 5 ml. of this inoculum by each of the following routes of administration; intravenous, intraperitoneal and intranasal. Clinical examinations were made and temperatures were taken daily for 2 weeks prior to inoculation and for 6 weeks postinoculation. At this time the pigs were killed and lung specimens were collected for histological examination. Lung, heart surface, peritoneal cavity and nasal turbinates were cultured on horse-blood agar, PPLO agar (Chanock et al., 1962a), and beef heart infusion agar with avian serum (Ross and Switzer, 1963) to determine the presence of microorganisms.

¹Dr. R. M. Chanock, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland.

RESULTS

Propagation Experiments

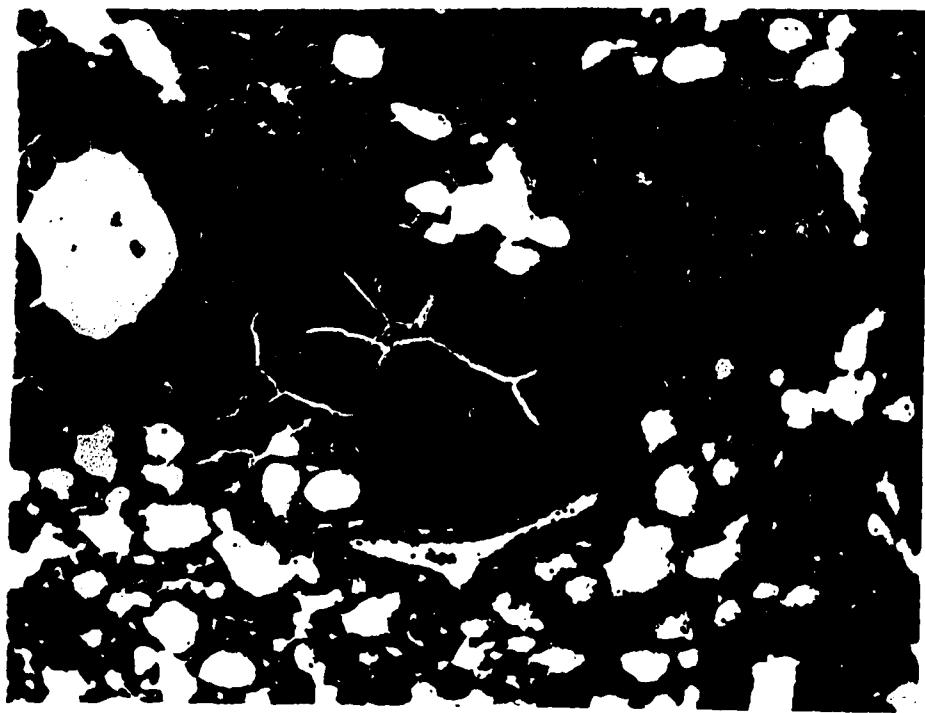
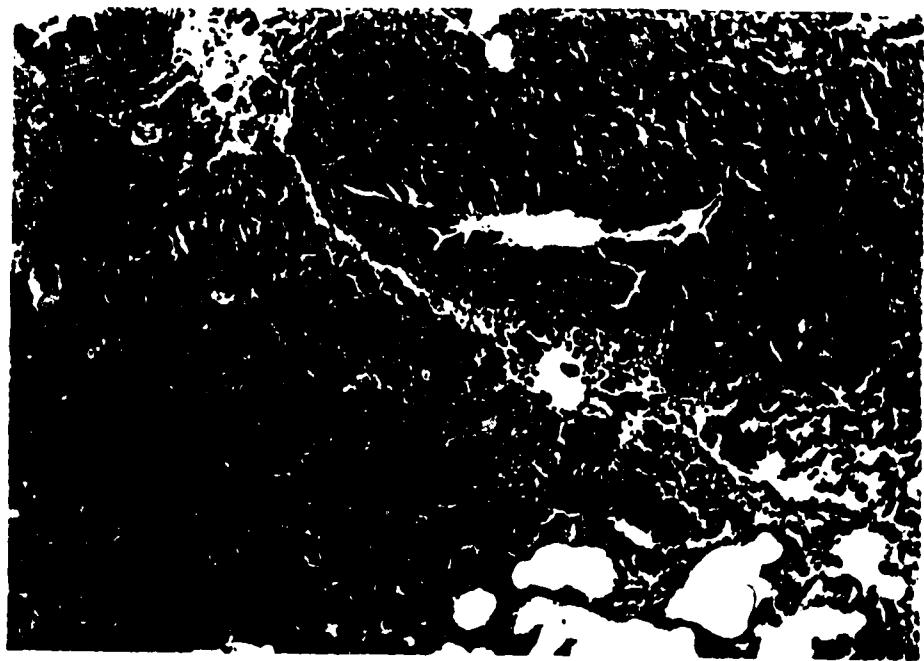
Cell cultures

The characteristic gross and microscopic lesions of virus pneumonia of pigs were found in 1 of 3 pigs necropsied 5 weeks after inoculation with 3rd serial passage lung cells which had been established from lesions of experimentally produced virus pneumonia of pigs. The gross lesions involved approximately 2 per cent of the lungs and were confined to the left apical lobe and the periphery of the left diaphragmatic lobe. Microscopic examination of the lungs revealed the characteristic peribronchiolar and perivascular lymphoid hyperplasia and marked thickening of the alveolar walls resulting from interstitial mononuclear leucocyte and fibroblast proliferation. Numerous giant cells were encountered in the areas of cellular proliferation. The microscopic lesions encountered in this pig are illustrated in Figures 2 and 3. Mild but definite microscopic lesions of virus pneumonia of pigs were encountered in the other 2 pigs inoculated with the same suspension.

No gross or microscopic lesions were found at necropsy of pigs inoculated with 7th and 8th serial passage swine lung cells established from pneumonic lesions. Similarly no lesions were present in pigs which were inoculated with normal serial passage swine lung cells which had been

Figure 2. Peribronchiolar lymphoid hyperplasia and alveolar interstitial thickening with fibroblast proliferation and giant cell formation. Lesions from pig inoculated with 3rd serial passage cell cultures established from pneumonic lung. Hematoxylin and eosin stain. X 100.

Figure 3. Peribronchiolar and perivascular lymphoid hyperplasia and alveolar interstitial thickening with fibroblast proliferation. From pig inoculated with 3rd serial passage cell cultures established from pneumonic lung. Hematoxylin and eosin stain. X 100.



inoculated with pneumonic lung suspension.

Three pigs were inoculated with a suspension prepared from the 8th serial transfer of virus pneumonia of pigs agent in serial passage swine lung cells grown at 37 C in a 95 per cent O_2 , 5 per cent CO_2 environment. The pigs were examined for lesions of virus pneumonia at necropsy 3 weeks later, but no gross or microscopic lesions were found. No lesions were found in 3 control pigs inoculated with a suspension of normal serial passage swine lung cells.

Artificial media

An organism was successfully propagated in Hanks' balanced salt solution with 0.5 per cent LH, 0.01 per cent yeast extract, 200 units/ml. penicillin, and 20 per cent heat-inactivated swine serum. A distinct growth spiral could be demonstrated 2 days after inoculation by gently shaking the tubes. Serial passages at 2 to 5 day intervals were performed and it was found that transfer at 3 or 4 day intervals was optimal. After 3 serial passages very poor growth was observed, but by the 7th transfer adaptation appeared to have occurred and distinct growth could thereafter be demonstrated from 2 days postinoculation onwards.

Giemsa stained preparations of the organisms were examined microscopically. Extremely small bluish-purple coccoid and cocco-bacillary organisms measuring from 300 to 500 μ predominated, but ring forms measuring up to 2μ in diameter

were occasionally observed. No change in the morphology of the organisms resulted from serial passage in the absence of penicillin. The characteristic appearance of the organisms is illustrated in Figures 4, 5, and 6. Figures 7 and 8 illustrate the appearance of Mycoplasma hyorhinis and Mycoplasma granularum stained with Giemsa. Orcein stained the organisms very densely, but too much stain deposit remained on the slides after washing, and Giemsa was considered the stain of choice. The organisms were found to be weakly Gram negative.

Successful colony growth was observed on agar plates prepared with Hanks' growth medium and inoculated with a Micrococcus sp. nurse colony in addition to the virus pneumonia of pigs inoculum. Distinct satelliting adjacent to the nurse colony was observed. No enhancement of colony growth was observed in agar media containing the 5 per cent lung extract. Very slow growth was observed on the same agar media in the absence of the nurse colony, the first colonies being visible after 6 days incubation, whereas with the nurse colony distinct colonies were seen after 2 days.

Direct isolation of the organisms from pneumonic lung suspensions was achieved in addition to the colony growth observed when agar surfaces were inoculated with the organisms grown in artificial media. The appearance of the colonies is illustrated in Figures 9 and 10. After 2 days very small round colonies were observed, and on the 3rd day distinct central raised areas as illustrated in Figures 9 and 10 were

Figure 4. Cluster of coccoid organisms in sediment from 7th serial passage culture of pneumonia agent in cell-free media. Giemsa stain. X 970.

Figure 5. Coccoid organisms in sediment from 7th serial passage culture. Giemsa stain. X 970.

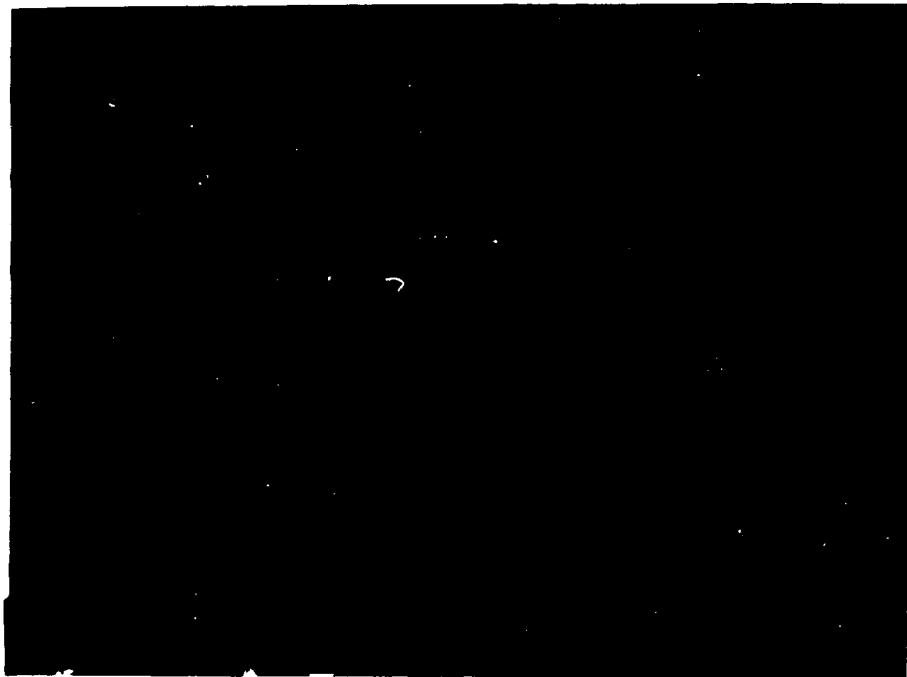
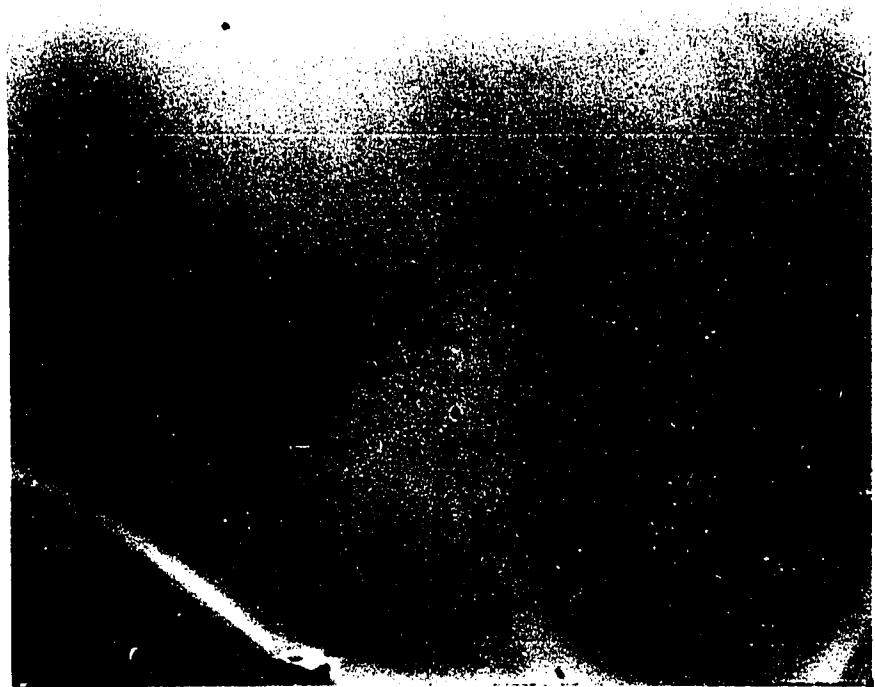


Figure 6. Clusters of bluish-purple coccoid to cocco-bacillary organisms and numerous ring forms in sediment from 7th serial passage culture of pneumonia agent in cell-free media. Giemsa stain. X 1250.

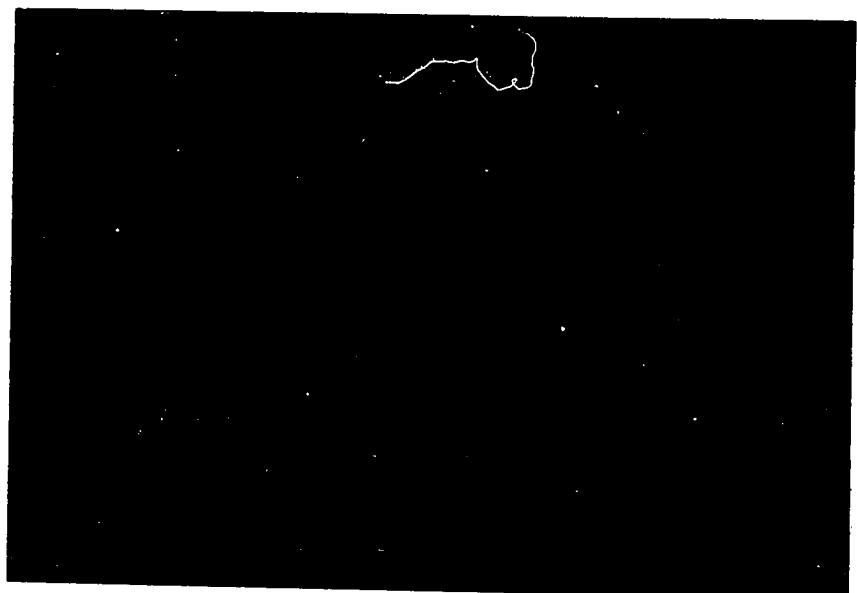


Figure 7. Sediment from culture of Mycoplasma hyorhinis.
Giemsa stain. X 1250.

Figure 8. Sediment from culture of Mycoplasma granularum.
Note dense-staining granules. Giemsa stain.
X 1250.

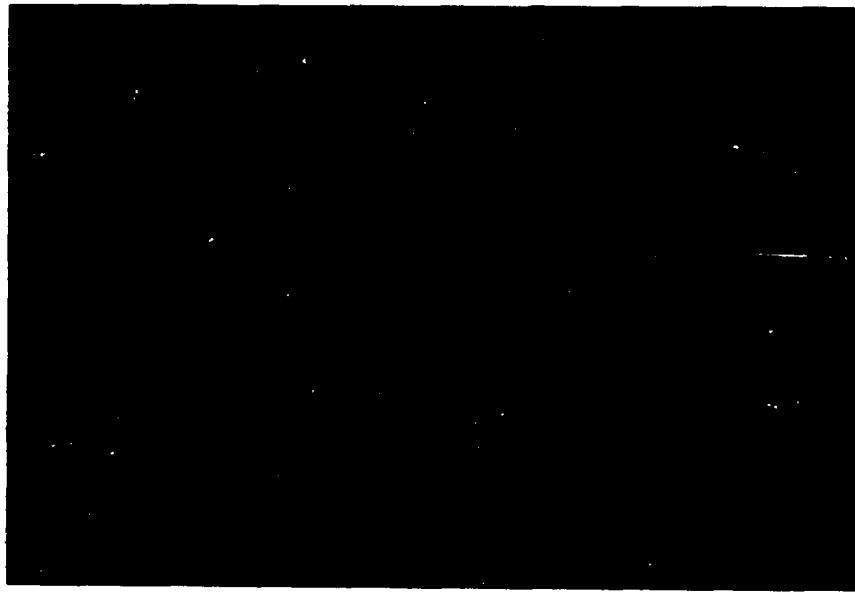
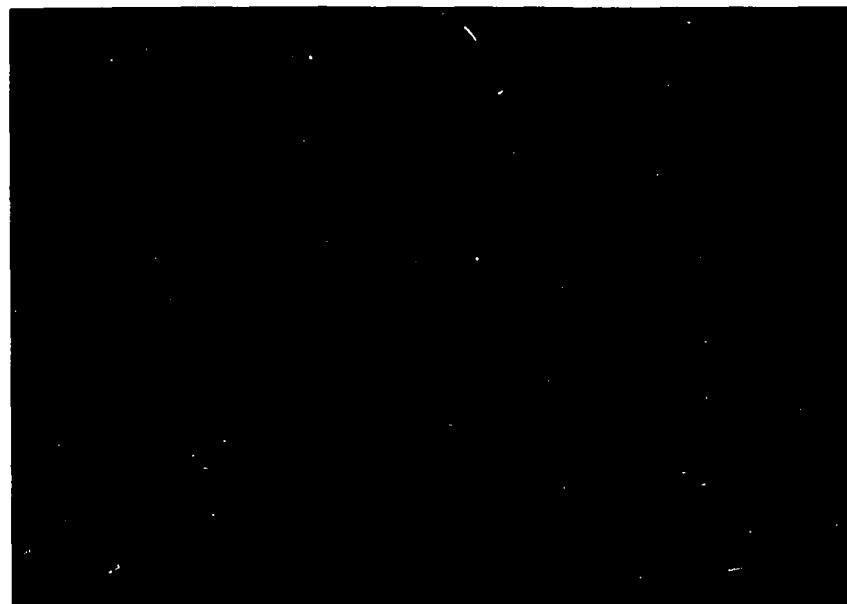


Figure 9. Colonies on surface of agar inoculated with suspension of pneumonic lung 3 days previously. Note distinct central raised areas on larger colonies.
X 35.

Figure 10. Colonies with distinct central raised areas.
Higher magnification of culture illustrated in
Figure 9. X 70.



observed. At this stage the largest colonies observed measured between 50 and 80μ . On the 6th day postinoculation some increase in size of colonies was observed, the largest measuring 100μ in diameter. The central raised area became more distinct as the colonies aged, and the surfaces of the colonies appeared to become somewhat rougher. The largest colony observed after 10 days growth measured 100μ in diameter.

Nine pigs were inoculated with the agent grown in artificial media, 3 pigs were inoculated with a mixed culture of the virus pneumonia of pigs agent and Mycoplasma hyorhinis, and 3 control pigs received the uninoculated Hanks' growth medium. No gross or microscopic lesions of virus pneumonia of pigs were observed in the control group of pigs or in the 3 pigs inoculated with the mixed culture when they were necropsied 21 days postinoculation. The characteristic gross lesions of virus pneumonia of pigs were observed in 3 of the 6 pigs which were inoculated with 7th serial passage of the organism in artificial medium. The lesions involved approximately 3 to 6 per cent of the lungs and were present in the right cardiac lobe in each case, and in addition, in the right apical, left cardiac and intermediate lobes in one case. The characteristic appearance of the gross lesions is illustrated in Figures 11 and 12. The appearance of the lungs of a control pig injected with uninoculated growth medium is illustrated in Figure 13. All 3 pigs inoculated with the 8th passage level of the organism in artificial medium were found

Figure 11. Ventral view of lungs with gross pneumonic lesions in apical, cardiac, diaphragmatic and intermediate lobes. From pig inoculated with 7th serial passage culture of organism grown in cell-free media.

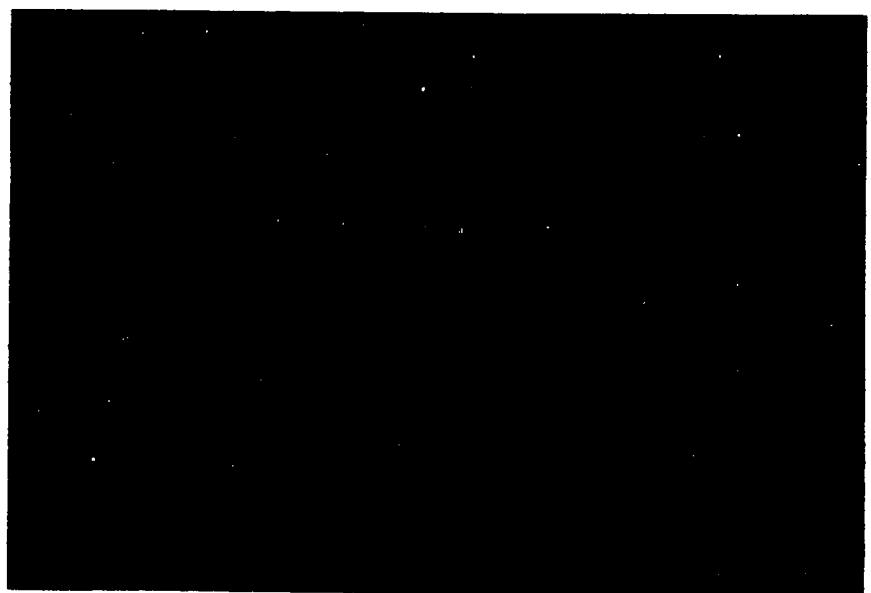
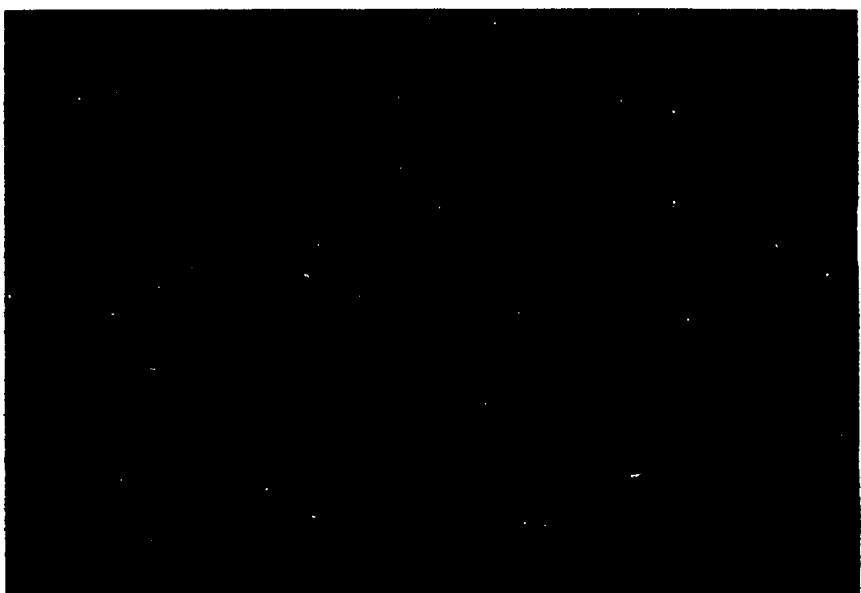
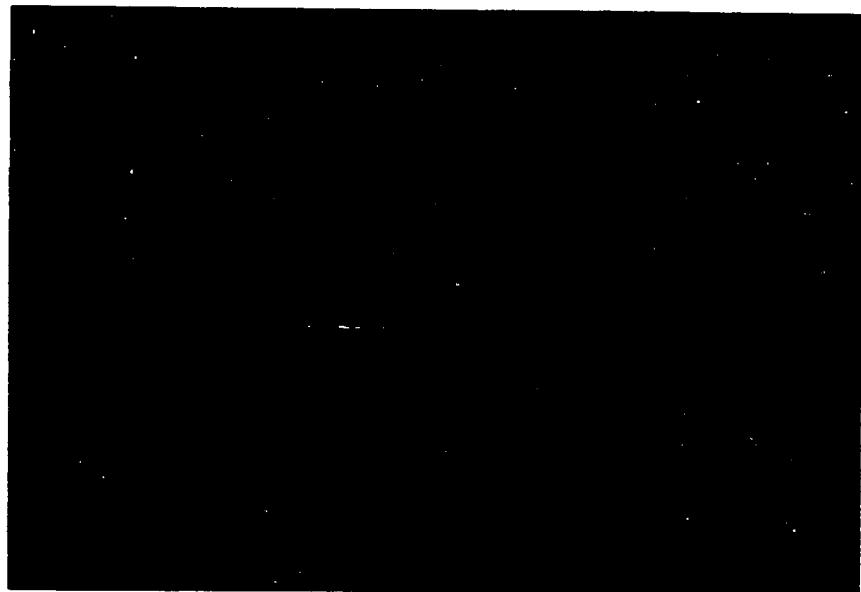


Figure 12. Dorsal view of lungs illustrated in Figure 11.
Note pneumonic lesions in right and left car-
diac lobes, right apical lobe and anterior edge
of left diaphragmatic lobe.

Figure 13. Normal lungs from control pig inoculated with un-
inoculated growth medium.



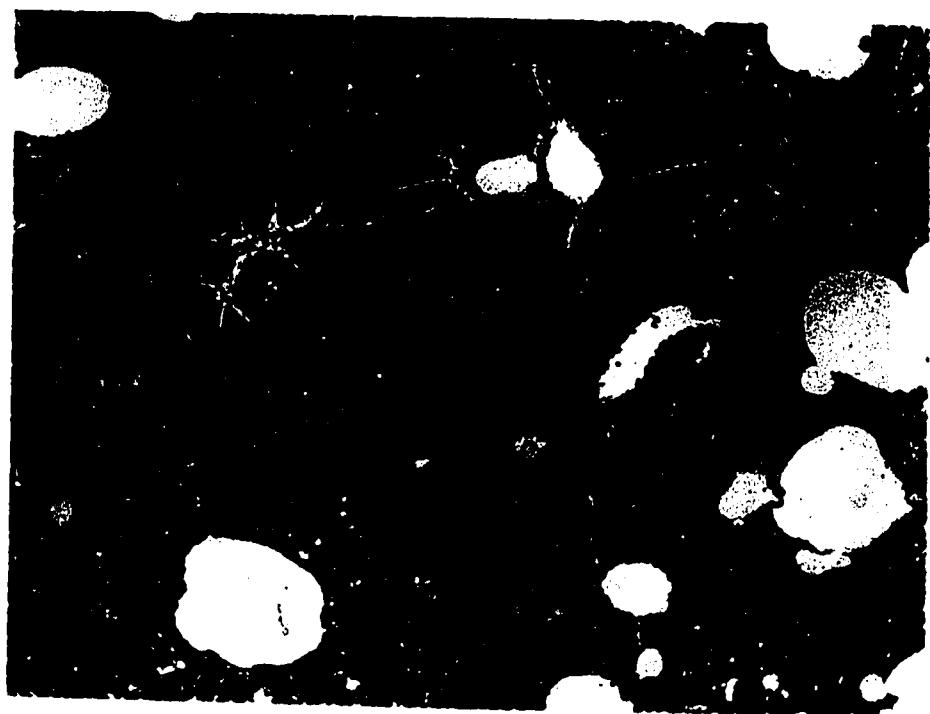
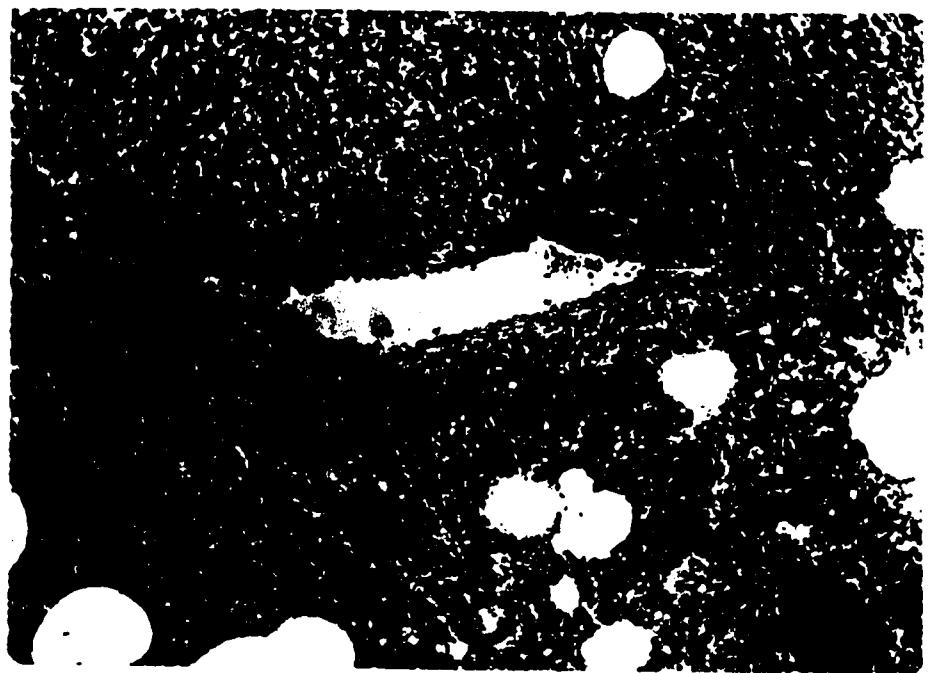
to have characteristic gross pneumonic lesions at necropsy. The lesions involved from 3 to 8 per cent of the lungs, the intermediate lobe being affected in each case, the right and left cardiac lobes in 2 out of the 3 pigs, and the anterior margin of the right diaphragmatic lobe in one case.

The characteristic microscopic lesions of virus pneumonia of pigs were encountered in 7 of the 9 pigs inoculated with the agent grown in artificial medium. The perivasicular and peribronchiolar accumulation of mononuclear cells was prominent and marked infiltration and resultant thickening of the alveolar walls was observed. Giant cells were occasionally encountered. The lesions illustrated in Figures 14 and 15 are typical of those encountered in each of the 7 affected pigs. Minute organisms were demonstrated in Giemsa-stained touch preparations prepared from freshly-cut surfaces of pneumonic lesions.

The organism was successfully reisolated in Hanks' broth and agar media from lesions collected at necropsy. The colony growth observed on plates inoculated with pneumonic lung suspensions is illustrated in Figures 9 and 10. No growth of Mycoplasma spp. or other organisms was observed in beef heart infusion-turkey serum medium inoculated with the same suspensions of pneumonic lung. No Bordetella bronchiseptica, Pasteurella multocida, Haemophilus spp. or Streptococcus spp. were isolated from the lesions.

Figure 14. Perivascular lymphoid hyperplasia and alveolar interstitial thickening with fibroblast proliferation and mononuclear cell infiltration. Lesions from pig infected with 7th serial passage culture of pneumonia agent in cell-free media. Hematoxylin and eosin stain. X 100.

Figure 15. Pronounced peribronchiolar and perivasculat lymphoid hyperplasia and alveolar interstitial thickening. Note occlusion of alveolar spaces. Lesions from pig inoculated with 7th serial passage culture of pneumonia agent in cell-free media. Hematoxylin and eosin stain. X 100.



Repeated attempts were made to isolate an agent from pneumonic lung using the swine Mycoplasma medium, various modifications of this medium, several avian Mycoplasma media, and the medium used for propagation of the human pathogen Mycoplasma pneumoniae. Broth and agar forms of each of these media were used, but no growth of organisms could be detected. Addition of a Micrococcus sp. nurse colony did not result in growth of the organisms on these media.

Chicken embryos

No consistent mortality pattern was observed in repeated attempts to propagate the agent of virus pneumonia of pigs in the yolk-sacs of embryonated hens eggs. Occasional sporadic deaths were observed, but on sub-passage of material from these eggs, no mortality occurred. Blind passages to the 6th passage level produced no change in this pattern. No lesions were observed on examination of chicken embryos 7 days post-inoculation.

Microscopic examination of Giemsa and Macchiavello stained yolk-sac smears revealed no microorganisms.

Three pigs which had been inoculated with a pooled 2nd and 6th serial passage embryo and yolk-sac suspension were necropsied 3 weeks postinoculation. No gross or microscopic lesions of virus pneumonia of pigs were observed.

No lesions were found in embryonated eggs which had been inoculated with a suspension of pneumonic lung by the

chorio-allantoic membrane route of inoculation. No mortality was observed in eggs infected in this way.

Mice

No pneumonic lesions were elicited in 21 day-old mice inoculated intranasally with a virus pneumonia of pigs lung suspension, and blind passage of mouse lung did not produce pneumonic lesions.

One group of mice injected intranasally with pneumonic lung suspension when 1 day old became sick 14 days postinoculation and all but 4 died. The 4 remaining sick mice were killed on the 20th day, but no gross or microscopic lesions could be demonstrated. The lungs were collected, pooled with lungs from other inoculated mice, and serially passaged in mice. No further mortality or gross or microscopic lesions were encountered.

Visualization Studies

Touch preparations

Extremely small coccoid to cocco-bacillary organisms and occasional ring forms were seen on microscopic examination of Giemsa-stained touch preparations prepared from freshly cut surfaces of pneumonic lung. They stained dark bluish-purple with Giemsa stain, and were just within the limits of resolution of the light microscope. Clusters of the organisms were most often encountered lying extracellularly closely

associated with cells. They occasionally appeared to be situated on the surface of cells, and definite intracellular aggregations were also encountered. Prolonged examination of touch preparations was often necessary before the organisms were found since they appeared to occur in definite foci. Once a focus was found, numerous clusters were usually demonstrable.

The organisms were encountered in touch preparations from pigs necropsied 3 to 5 weeks postinoculation, but were not demonstrable in touch preparations prepared from lesions in pigs necropsied 8 weeks postinoculation.

The size of the organisms as determined by the use of an eye-piece micrometer varied from less than 300 to 500 $\text{m}\mu$ in diameter. Those occurring intracellularly appeared to be enclosed in large vacuoles, and marked distension of infected cells was observed. This phenomenon is illustrated in Figure 16. The organisms which appeared supracellularly are illustrated in Figure 17. Their supracellular occurrence was determined by focussing up and down through the focal depth of the cell, thus accentuating the fact that they were situated on the outside curvature of the cells and not in one plane as they appeared to be when situated intracellularly. The variation in the size of the clusters of organisms encountered in touch preparations is illustrated in Figures 18, 19, 20, and 21. The slightly elongated cocco-bacillary form of the

Figure 16. Large cluster of coccoid organisms in cytoplasm of cell. Touch preparation from pneumonic lesion. Giemsa stain. X 970.

Figure 17. Organisms on outside surface of cell. Touch preparation from pneumonic lesion. Giemsa stain. X 970.

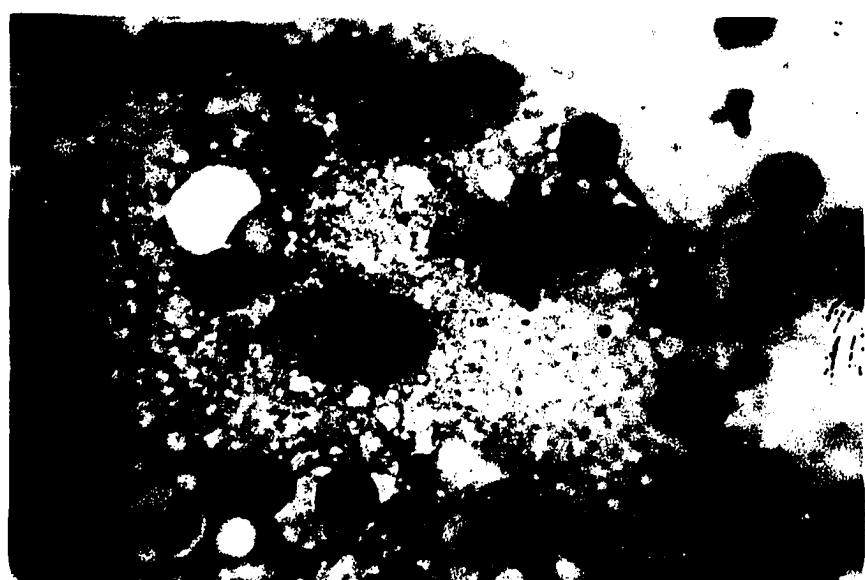


Figure 18. Extracellular organisms showing tendency toward cocco-bacillary form. Touch preparation from pneumonic lesion. Giemsa stain. X 970.

Figure 19. Large extracellular accumulation of coccoid organisms with a few distinct ring forms. Touch preparation from pneumonic lesion. Giemsa stain. X 970.



Figure 20. Small clusters of extracellular coccoid organisms. Touch preparation from pneumonic lesion. Giemsa stain. X 970.

Figure 21. Very large accumulation of coccoid organisms closely associated with lymphocytes and large macrophage. Touch preparation from pneumonic lesion. Giemsa stain. X 970.



organism is illustrated in Figure 18, and the ring forms in Figure 19. The organisms were not observed in touch preparations prepared from normal lung.

Attempted isolation of bacteria, especially Mycoplasma hyorhinis and M. granularum from lungs from which the touch preparations were made, was consistently negative.

Histological sections

The lesions encountered in pigs experimentally infected with strain 11 of the agent of virus pneumonia of pigs varied considerably. Perivasicular and peribronchiolar lymphoid hyperplasia was consistently encountered, and specimens were not considered positive unless this change was observed. The degree of hyperplasia was quite variable, being very pronounced and practically the only lesion present in some cases (Figures 58 and 59), while in other instances it was associated with marked alveolar interstitial thickening, septal cell proliferation and neutrophil infiltration (Figures 51 and 61). Interstitial giant cell formation was frequently encountered in lesions from pigs experimentally infected with the agent. This phenomenon was not encountered in pigs infected with the early pig passage levels of the agent, but after the 4th serial passage, giant cells were frequently demonstrable. The typical appearance of the giant cells is illustrated in Figures 2, 22 and 50. An increase of intra-alveolar connective tissue was usually associated with the

giant cell formation.

Catarrhal bronchiolitis was occasionally observed in early cases of virus pneumonia of pigs, but in most cases the bronchiolar epithelium appeared normal. Sub-epithelial infiltration of lymphocytes in the bronchioli was occasionally observed (Figure 57).

The small coccoid organisms which were observed in touch preparations were also demonstrated in sections of pneumonic lung stained with Giemsa. They were most often encountered on the surface of the bronchiolar epithelium, but could also be demonstrated in the alveoli and rarely in swollen septal cells. The organisms were not found in lesions of more than 5 weeks duration, but were quite frequently encountered in sections prepared from lesions collected 3 weeks postinoculation. Their characteristic appearance is illustrated in Figures 23 and 24.

Cell cultures

Cell cultures which were established from pneumonic lung, and which after 3 serial passages were found to be infective by pig inoculation, were stained with Giemsa and examined microscopically. Occasional cells were found to contain the coccoid organisms in their cytoplasm or on the surface of the cells. The organisms were not numerous, but when encountered could be easily visualized. The characteristic appearance of normal and infected cell sheets is illustrated in Figures 25, 26, 27, and 28. Normal serial passage swine lung cells

Figure 22. Giant cell with peripheral nuclei. Note pronounced alveolar interstitial thickening with mononuclear cell infiltration. Hematoxylin and eosin stain. X 430.

Figure 23. Cocco-bacillary organisms on surface of bronchial epithelium. Section of pneumonic lesion. Giemsa stain. X 900.

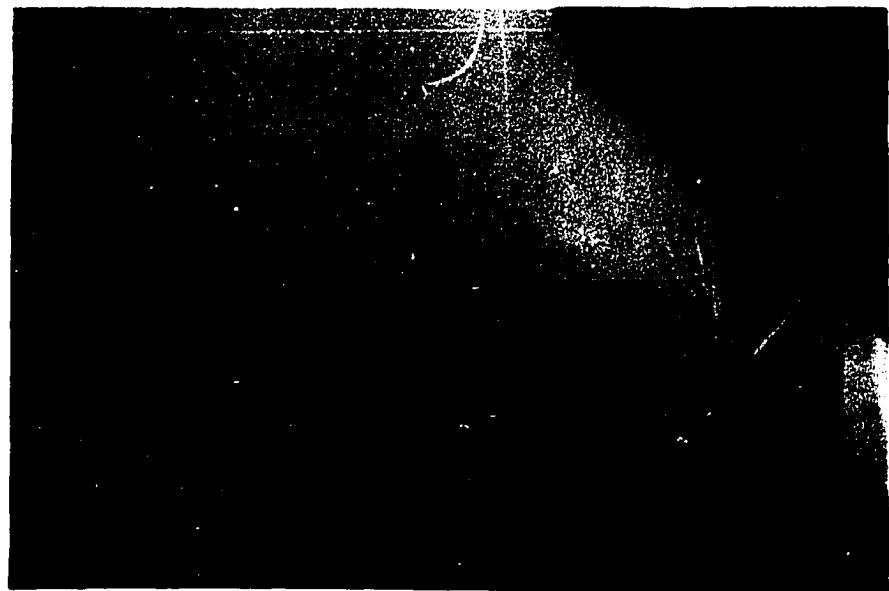


Figure 24. Clumps of coccoid organisms on surface of bronchiolar epithelium. Section of pneumonic lesion. Giemsa stain. X 900.

Figure 25. Serial passage swine lung cell cultures established from normal lung. Giemsa stain. X 100.

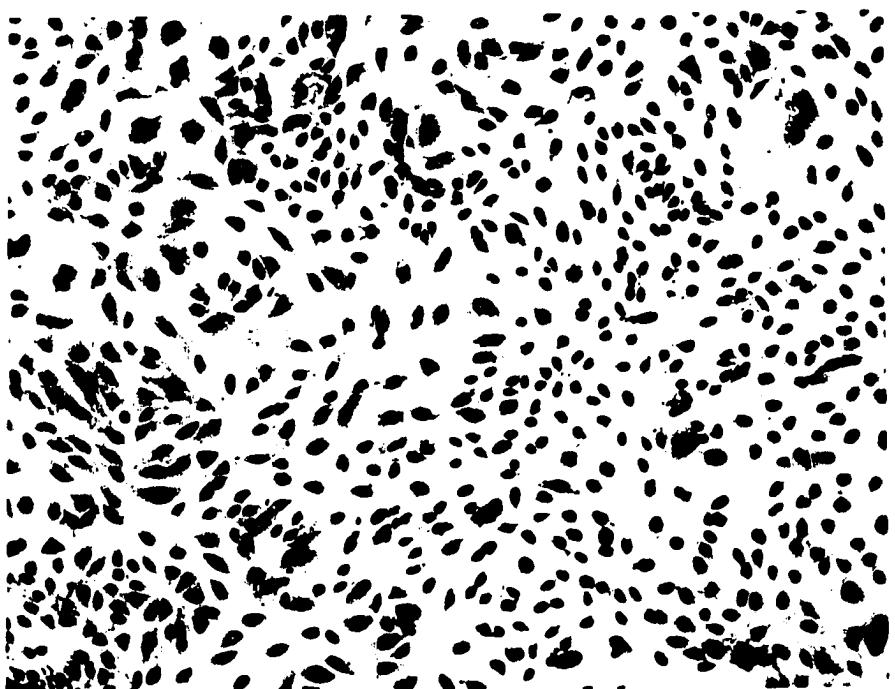


Figure 26. Higher magnification of cell sheet illustrated in Figure 25. Giemsa stain. X 430.

Figure 27. Coccoid organisms in serial passage swine lung cell cultures infected with pneumonic lung suspension. Giemsa stain. X 430.

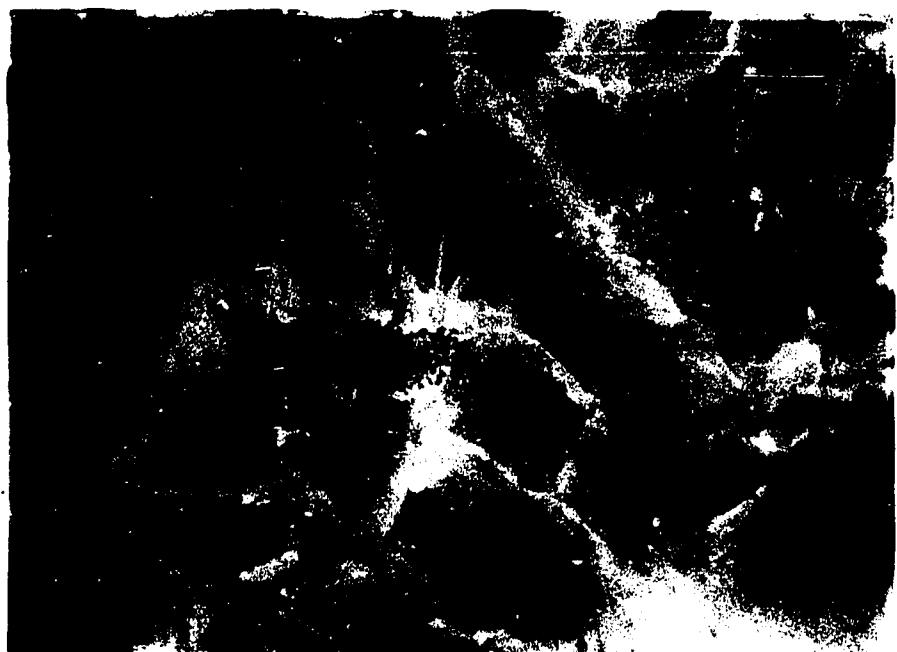


Figure 28. Extracellular clump of coccoid organisms in serial passage swine lung cells established from pneumonic lung. Giemsa stain. X 1100.



inoculated with filter-clarified pneumonic lung suspension were also found to contain the organisms (Figure 27).

Acridine orange preparations

The appearance of cell cultures of normal swine lung stained with acridine orange is illustrated in Figures 29 and 30. The reddish-orange staining is characteristically associated with the RNA of the nucleoli and the ribosomes, while the green-staining DNA is confined to the nucleus. In cell cultures established from pneumonic lung, some deviations from the normal staining pattern were observed. Cells were occasionally found to contain dense reddish-orange staining bodies assumed to represent focal accumulation of RNA. This phenomenon is illustrated in Figures 31 and 32. Other cells were found to contain extranuclear green-staining bodies assumed to be accumulations of DNA (Figures 33 and 34). Both the red and the green bodies varied considerably in size, and were not encountered in all cell cultures of pneumonic lung.

The reddish bodies were frequently encountered in touch preparations prepared from pneumonic lung and stained with acridine orange. They were not present in preparations of normal lung.

Electron microscopic observations

In an effort to gain familiarity with the ultrastructure of the normal pig lung the lungs of 3 respiratory

Figure 29. Normal swine lung cell culture. Acridine orange stain. X 600.

Figure 30. Higher magnification of normal swine lung cell culture illustrated in Figure 29. Acridine orange stain. X 1100.

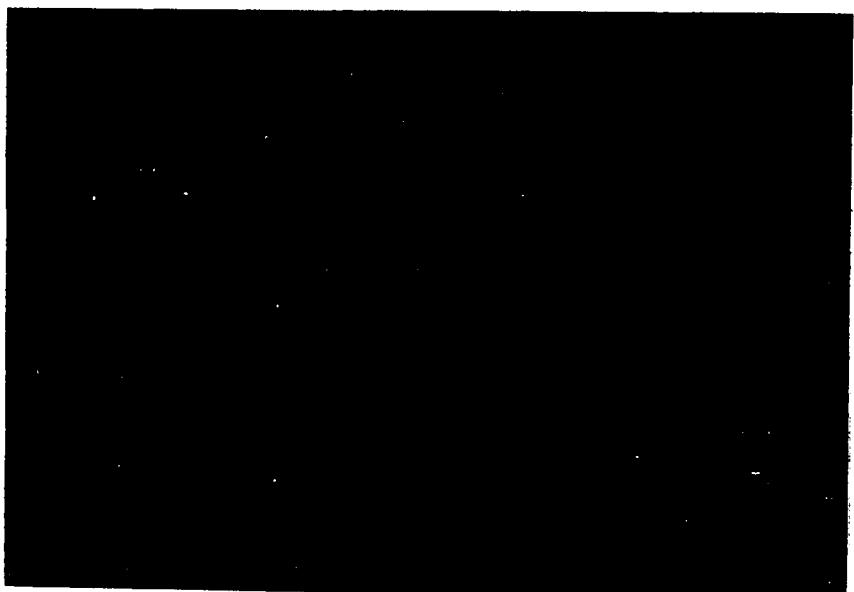
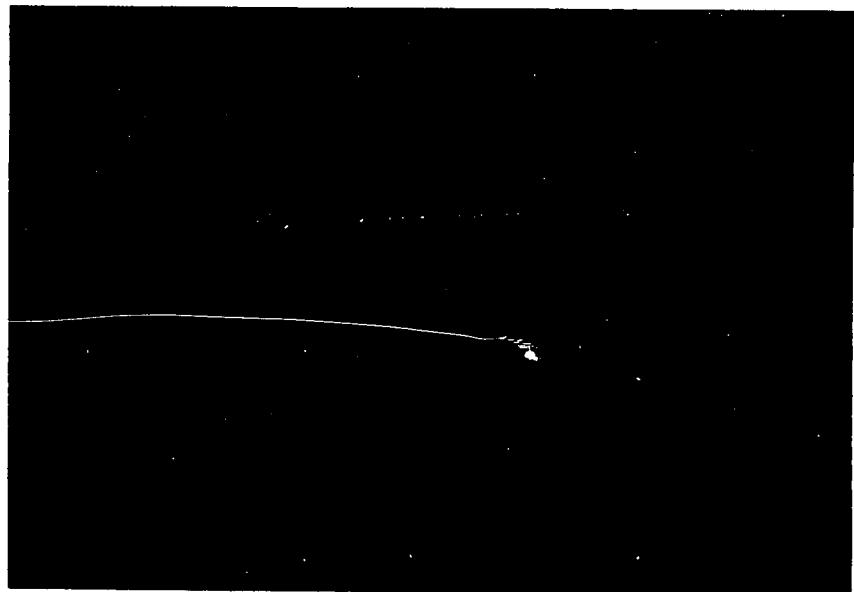


Figure 31. Cell culture established from pneumonic swine lung. Note reddish-orange bodies in cell cytoplasm, one of them indenting nucleus. Acridine orange stain. X 1100.

Figure 32. Cell culture established from pneumonic lung. Several reddish-orange inclusions. Acridine orange stain. X 1100.

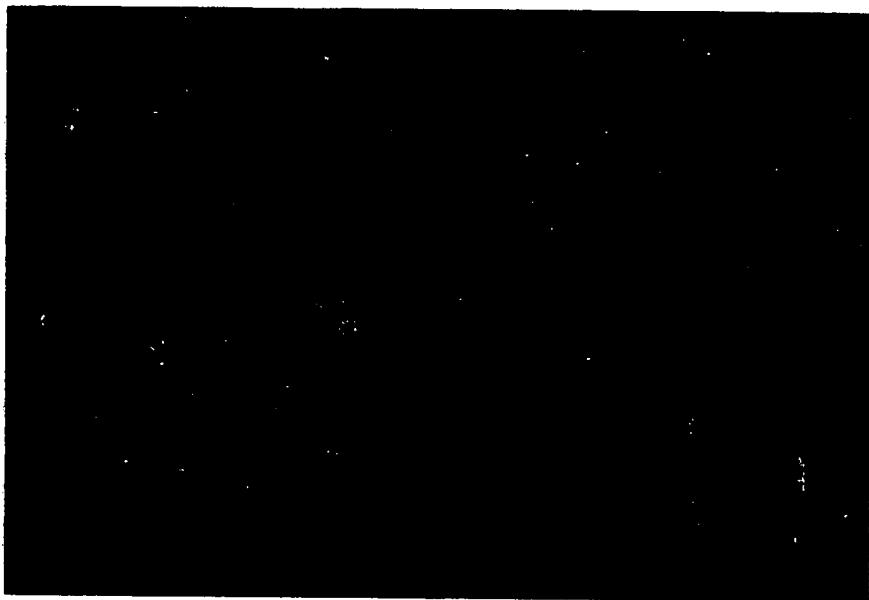
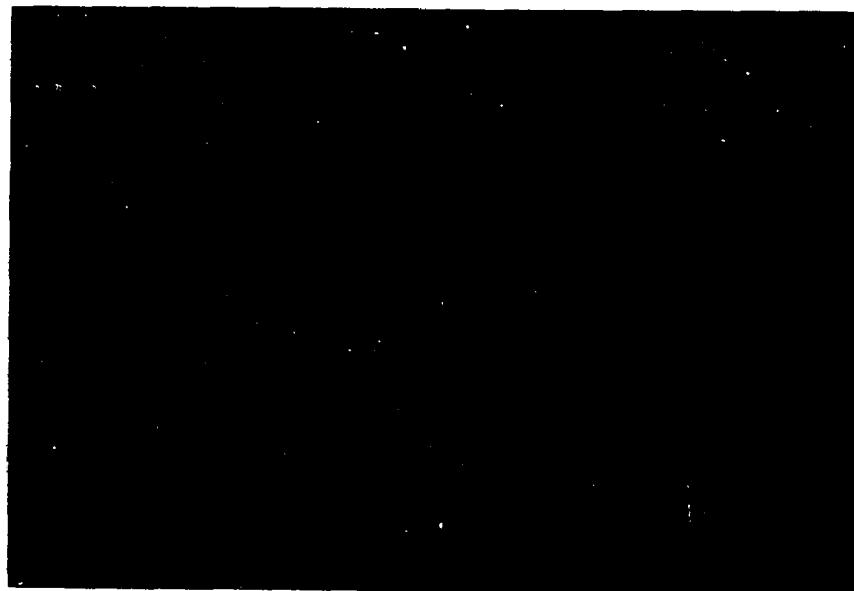


Figure 33. Cell culture of pneumonic lung with several greenish cytoplasmic inclusions. Acridine orange stain. X 1100.

Figure 34. Cell culture of pneumonic lung with green intracytoplasmic inclusion. Acridine orange stain. X 1100.



disease free pigs were examined with the electron microscope. A continuous alveolar lining epithelium was demonstrated. Two types of cells were found to be involved in the formation of the alveolar epithelium. The predominant cell type characteristically possessed markedly attenuated cytoplasm with an average thickness of less than 0.5μ . Mitochondria and other cytoplasmic organelles were infrequently encountered. These cells rested on well-defined basement membranes and formed the main part of the alveolar lining. They will be referred to as "alveolar epithelial lining cells" consistent with the terminology proposed by Omar (1964). The characteristic appearance of the alveolar epithelial lining cells is illustrated in Figures 35, 36, 37, and 38. The second type of cell was roughly polygonal in shape, and was characteristically encountered in recesses of the alveolar lumina. These cells did not possess attenuated cytoplasm, but contained numerous mitochondria, lamellar bodies and other cytoplasmic organelles. Microvilli were often present on the free surfaces of these cells. They were very seldom found lying free in the alveolar spaces in the normal lung. These cells will be referred to as "septal cells" as suggested by Omar (1964). The typical appearance of a septal cell containing numerous lamellar bodies (Bensch et al., 1964) is illustrated in Figure 39. "Intra-septal cells" (Omar, 1964) were occasionally encountered in the alveolar walls (Figures 37 and 38).

Figure 35. Normal swine lung. Structure of wall separating capillary lumen (C) from alveolar space (A). Note attenuated cytoplasm of alveolar lining cell (EP), and overlapping at junction of capillary endothelial cells (EN). Basement membranes (BM) and caveolae intracellularis (arrows) are prominent. Uranyl acetate stain. X 50,400.



Figure 36. Normal swine lung. Cross-section of capillary containing thrombocyte (T). Structure of wall separating alveolar space (A) from capillary lumen (C) very distinct. Numerous caveolae intracellularis (arrows) and a few mitochondria (M) are in evidence. Uranyl acetate stain. X 50,400.



Figure 37. Normal swine lung. Note attenuated cytoplasm of alveolar epithelial lining cell (EP) containing mitochondrion (M). Several mitochondria are present in an intra-septal cell (IS). Uranyl acetate stain. X 26,400.

Figure 38. Normal swine lung. Note intra-septal cell with large nucleus (N) and mitochondria (M), and attenuated cytoplasm of alveolar epithelial lining cell (EP). Uranyl acetate stain. X 26,400.



Figure 39. Normal swine lung. Septal cell in recess of alveolar lumen (A). Note large centrally situated nucleus (N), mitochondria (M), lamellar bodies (L) and microvilli (MV). Uranyl acetate stain. X 26,400.



The ultrastructure of the walls separating the lumina of the capillaries from the alveolar spaces is illustrated in Figures 35 and 36. The characteristic alveolar epithelial lining cell resting on its basement membrane was separated from the basement membrane of the capillary endothelium by a space of matrix of varying width in which collagen fibrils were occasionally encountered. Numerous micropinocytotic vesicles were present in both the alveolar epithelial and capillary endothelial cells. Caveolae intracellularis were present on the free and attached sides of both types of cells. No fenestration of the endothelium was observed, and at the junction between adjacent cells, varying degrees of overlapping could be demonstrated.

Electron microscopic examination of lesions of virus pneumonia of pigs revealed a very marked increase in the number and size of septal cells. These cells were frequently encountered lying loose in the alveolar spaces sometimes closely associated with neutrophiles or lymphocytes. The cytoplasm of the septal cells appeared to be markedly distended and contained large numbers of mitochondria, ribosomes and occasionally osmiophilic membrane-bound bodies with electron-dense central regions and electron-opaque peripheries. These bodies were occasionally encountered lying free in the alveolar spaces (Figure 45). They varied in size from 200 to 350 m μ in longest diameter. The characteristic

morphology of these bodies is illustrated in Figures 40 and 41. The appearance of the distended free-lying septal cells is illustrated in Figure 42. The typical appearance of an attached septal cell of the type in which the osmiophilic bodies were encountered is illustrated in Figure 43.

Occasionally bodies somewhat larger than those described previously were found in the cytoplasm of the septal cells. These structures were membrane-bound and contained numerous small membrane-bound bodies giving them a vesicular appearance. The appearance of one of these vesiculated structures is illustrated in Figure 44.

What appeared to be an amorphous layer on the periphery of the alveolar spaces was observed in sections of pneumonic lungs (Figure 43). This material seemed to be associated with the distended septal cells and not the alveolar lining epithelium.

Cell cultures which were established from lesions of virus pneumonia of pigs, and which after 3 serial cell passages were shown by pig inoculation to be infective, were examined with the electron microscope. Numerous electron-dense bodies, some of them clearly membrane-bound, were encountered in the cytoplasm of some cells. These bodies measured from 200 to 450 μ in diameter, occasionally contained electron-dense "nucleoid" structures, and sometimes membranous whorls resembling the mesosomes of bacteria.

Figure 40. Pneumonic swine lung. Membrane bound body (arrow) in cytoplasm of swollen septal cell. Note the electron-dense central portion of the body surrounded by an electron-transparent zone. This body measured 300 $\text{m}\mu$ in its largest axis. A mitochondrion (M) and the nucleus of the cell (N) can be seen. Uranyl acetate stain. X 126,000.

Figure 41. Pneumonic swine lung. Several of the electron-dense membrane-bound bodies (arrows) lying adjacent to the nucleus (N) of the cell. Uranyl acetate stain. X 126,000.



Figure 42. Pneumonic swine lung. Distended septal cells (arrows), neutrophile (NE) and erythrocyte (E) in alveolar space (A). Uranyl acetate stain. X 7,200.

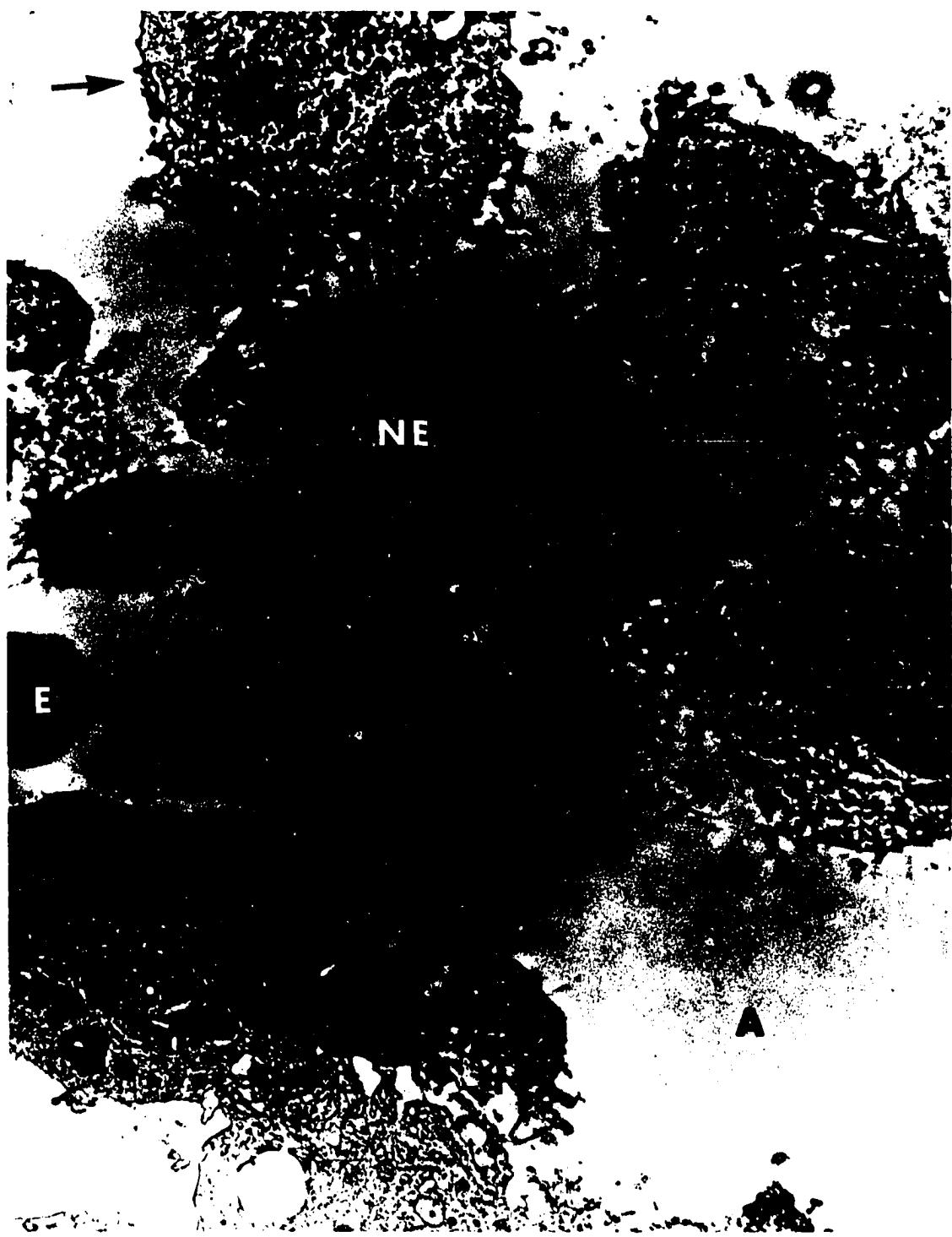
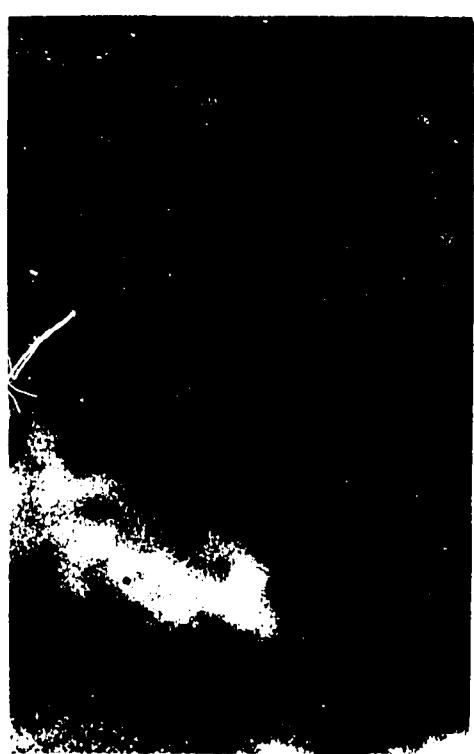
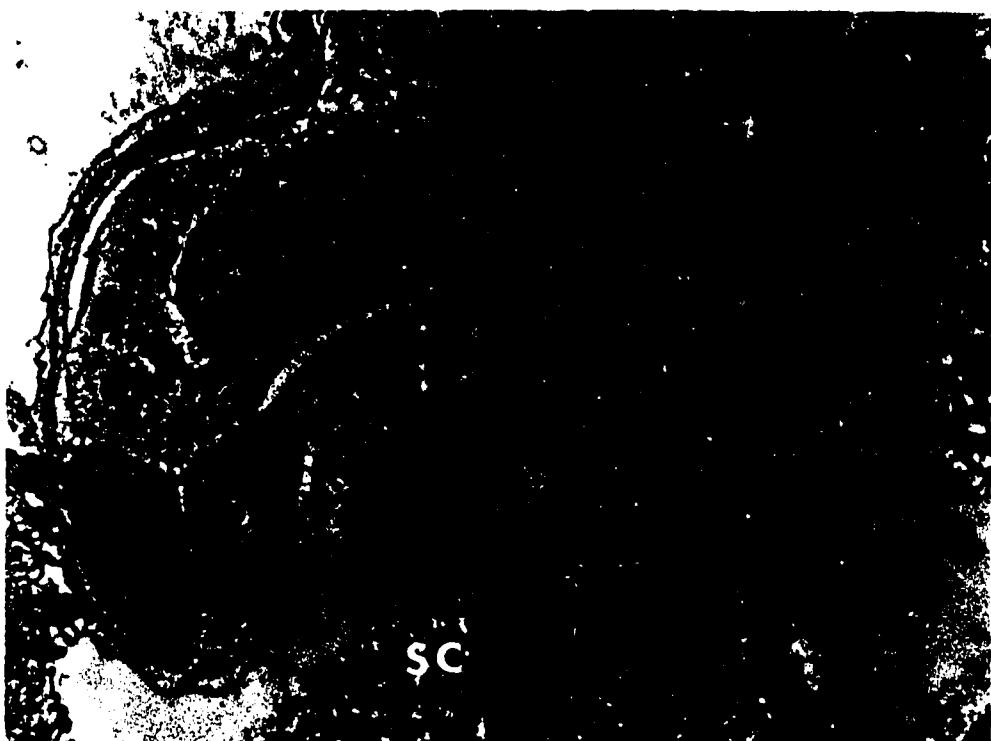


Figure 43. Pneumonic swine lung. Amorphous material (arrow) in alveolar space (A). Note adherence of this material to surface of attached septal cell (SC). Uranyl acetate stain. X 7,200.

Figure 44. (lower left) Pneumonic swine lung. Vesicular body (arrow) in cytoplasm of septal cell. Uranyl acetate stain. X 63,000.

Figure 45. (lower right) Pneumonic swine lung. Free-lying body (arrow) in alveolar space (A). Uranyl acetate stain. X 63,000.



These "finger-print-like" whorls were centrally or peripherally situated. The characteristic appearance of the bodies encountered in the cells is illustrated in Figures 46, 47, and 48. Occasionally these bodies appeared to be partially or completely enclosed within larger membrane-bound inclusions as shown in Figure 49. Osmophilic inclusions resembling those found in the virus pneumonia of pigs cell cultures were not encountered in cell cultures established from normal swine lung.

Filtration Studies

In the first filtration trial a virus pneumonia lung suspension was clarified through a membrane of 800 $\text{m}\mu$ APD, and was then divided into 4 aliquots, one of which served as a control while the other three were each passed through a membrane of 650 $\text{m}\mu$, 450 $\text{m}\mu$ or 300 $\text{m}\mu$ APD. Pigs inoculated with the filtrates were necropsied 30 days postinoculation, and examined for lesions of virus pneumonia of pigs. All 16 pigs had characteristic gross lesions involving from 1 to 18 per cent of the lungs. On microscopic examination, the typical perivascular and peribronchiolar lymphoid hyperplasia was encountered in each case. No bacteria or Mycoplasma spp. could be isolated from the lungs.

It was decided to include fowl-pox virus in the inoculum for the 2nd filtration trial. A preliminary size determination of the fowl-pox virus was performed using the 800 $\text{m}\mu$,

Figure 46. Cell culture established from pneumonic lung. Very distinct whorls (arrows) in electron-dense bodies. Nucleoid (N) prominent in body at lower right. Uranyl acetate stain. X 63,000.

Figure 47. Cell culture established from pneumonic lung. Numerous electron-dense bodies in cytoplasm. Note membranous whorl (arrow) and nucleoid (N). Uranyl acetate stain. X 63,000.



Figure 48. Cell culture established from pneumonic lung.
Note electron-dense body (arrow) with distinct
peripheral membrane. Uranyl acetate stain.
X 63,000.

Figure 49. Cell culture established from pneumonic lung.
Two membrane-bound bodies are partially enclosed
within large membrane-bound structure (arrow).
Golgi apparatus (G) and mitochondria (M) are
distinct. Uranyl acetate stain. X 63,000.

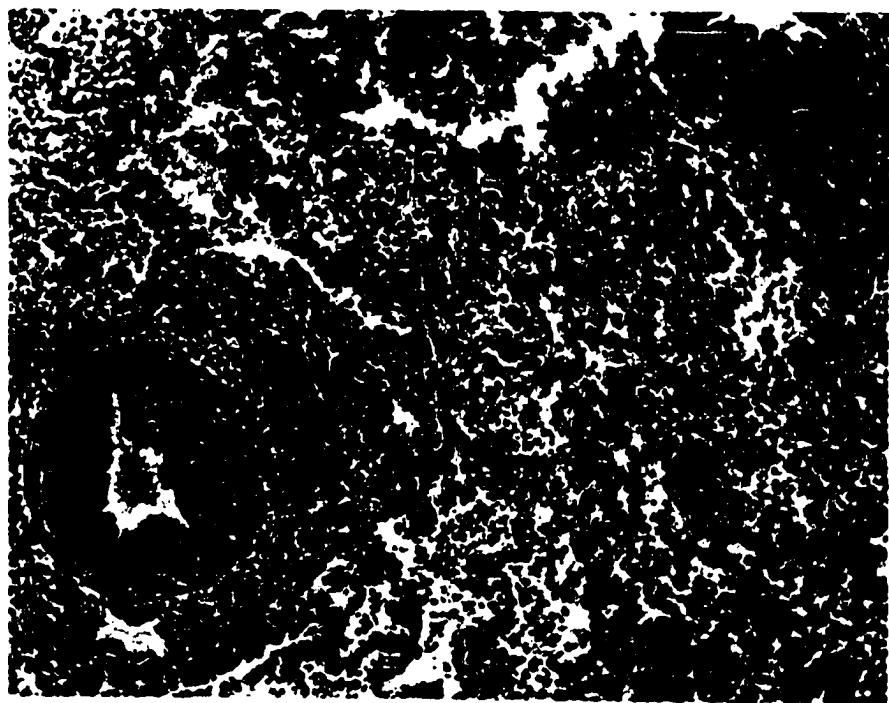


450 m μ , 300 m μ and 220 m μ APD membranes. Embryonated hens eggs inoculated onto the chorio-allantoic membrane with each of the filtrates were opened 7 days postinoculation and examined for pox lesions. Distinct, clearly-defined lesions were present on the membranes of eggs inoculated with the 800 m μ , 450 m μ and 300 m μ filtrates. No lesions were found on the membranes of the eggs inoculated with the 220 m μ filtrate.

Fowl-pox virus was added to the lung suspension to give a final dilution of 10^{-2} of the stock fowl-pox virus. The mixture was filtered through 800 m μ , 450 m μ , 300 m μ and 220 m μ membranes and inoculated into pigs and onto the chorio-allantoic membrane of 10 day-old embryonated eggs. One group of pigs was inoculated with fowl-pox virus alone. No pox lesions were encountered in the respiratory tracts of the pigs when necropsied 21 days postinoculation. All 3 pigs inoculated with the 800 m μ filtrate had characteristic gross and microscopic lesions of virus pneumonia of pigs. One of the 3 pigs receiving the 450 m μ filtrate had characteristic gross pneumonia, and on microscopic examination typical lesions were present in this pig. Typical gross lesions were also found in 1 of 3 pigs receiving the 300 m μ filtrate, and characteristic lesions were encountered on microscopic examination of the lungs of this pig. The microscopic lesions resulting from inoculation with the 450 m μ and 300 m μ filtrates are illustrated in Figures 50 and 51. No gross or microscopic

Figure 50. Peribronchiolar lymphoid hyperplasia, septal cell proliferation, neutrophil infiltration and giant cell formation. Lesions in pigs infected with 450 $\text{m}\mu$ filtrate. Hematoxylin and eosin stain. X 100.

Figure 51. Very pronounced peribronchiolar and perivascular lymphoid hyperplasia with septal cell proliferation and neutrophil infiltration. Lesions from pig infected with 300 $\text{m}\mu$ filtrate. Hematoxylin and eosin stain. X 100.



pneumonic lesions were encountered in 4 pigs inoculated with the 220 μm filtrate or in the 3 pigs inoculated with fowl-pox virus. The microscopic appearance of the lungs of pigs from the 2 negative groups is illustrated in Figures 52 and 53.

The eggs inoculated with the mixed fowl-pox and lung suspension filtrates were examined for lesions 7 days post-inoculation. Distinct pox lesions were present on the chorio-allantoic membranes of eggs inoculated with the 800 μm , 450 μm and 300 μm filtrates and with the unfiltered fowl-pox virus. No lesions were found in eggs inoculated with the 220 μm filtrate or with virus pneumonia of pigs lung suspension. The appearance of the membranes from eggs inoculated with the 300 μm and 220 μm filtrates is illustrated in Figure 54.

No bacteria or Mycoplasma spp. were isolated from lungs collected at necropsy, nor was fowl-pox virus isolated from these tissues.

The results of the 2 filtration trials are summarized in Table 1. The normal lungs of a pig receiving the 220 μm filtrate and the pneumonic lungs of a pig receiving the 800 μm filtrate are illustrated in Figures 55 and 56.

Serology

Agar-gel diffusion micro-precipitation tests

Virus pneumonia of pigs antigen, feline pneumonitis antigen, Bordetella bronchiseptica antigen and the appropriate

Figure 52. Lung from pig inoculated with 220 μ filtrate.
No lesions in evidence. Hematoxylin and eosin
stain. X 100.

Figure 53. Lung from pig inoculated with unfiltered fowl-pox
virus. No lesions observed. Hematoxylin and
eosin stain. X 100.

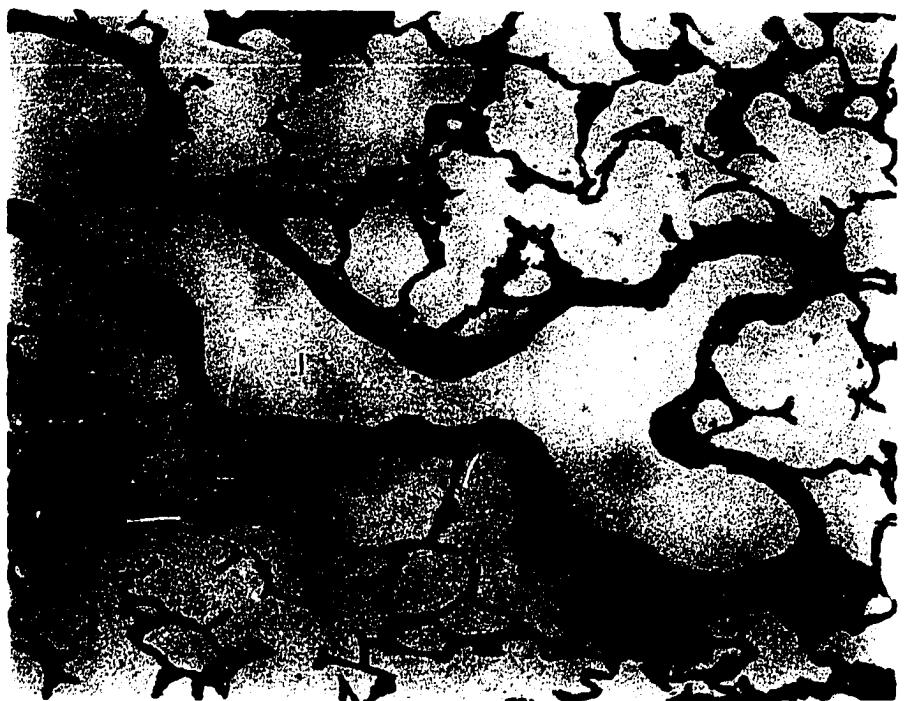
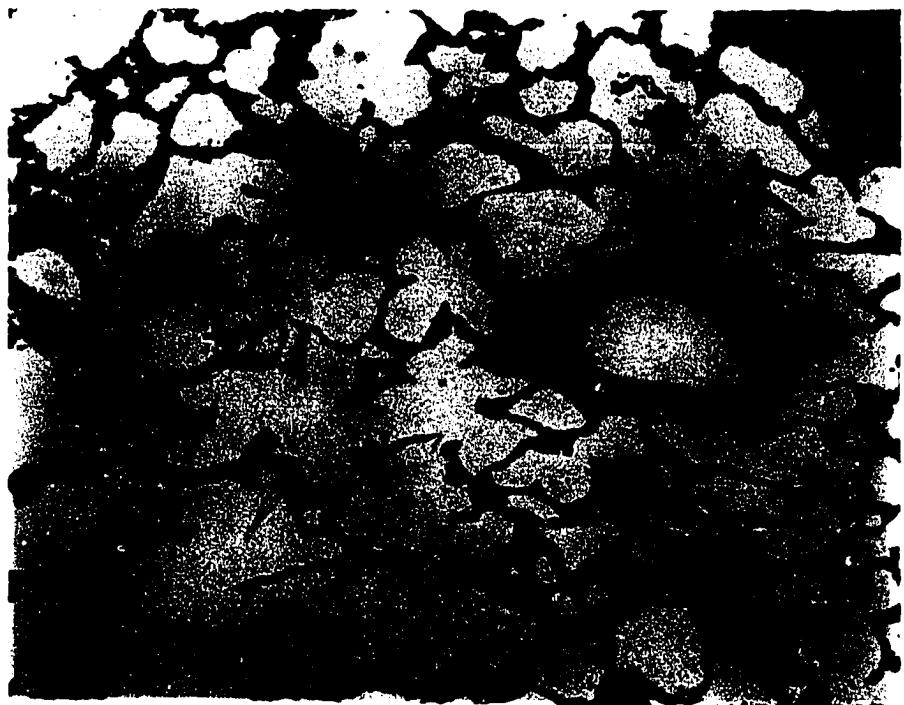


Figure 54. Comparison of chorio-allantoic membranes from embryonated eggs inoculated with 300 $m\mu$ and 220 $m\mu$ filtrates of mixture of pneumonic lung suspension and fowl-pox virus. Note congestion and distinct pox lesions in 300 $m\mu$ membranes.

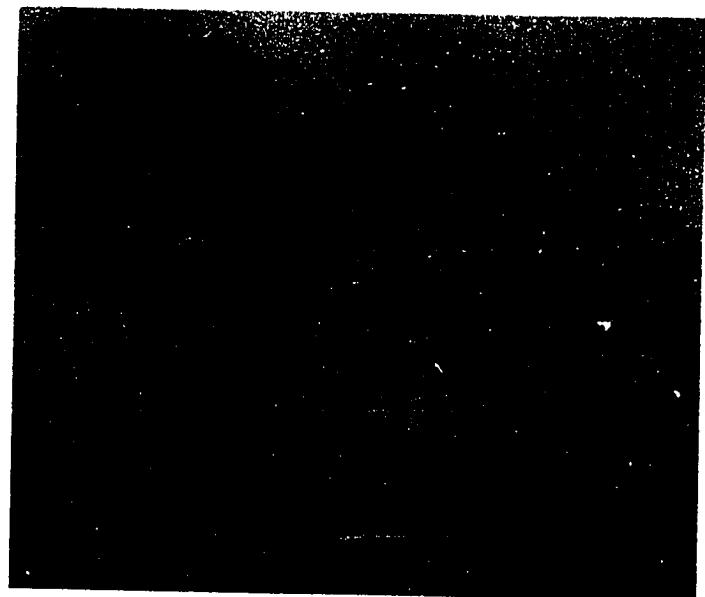
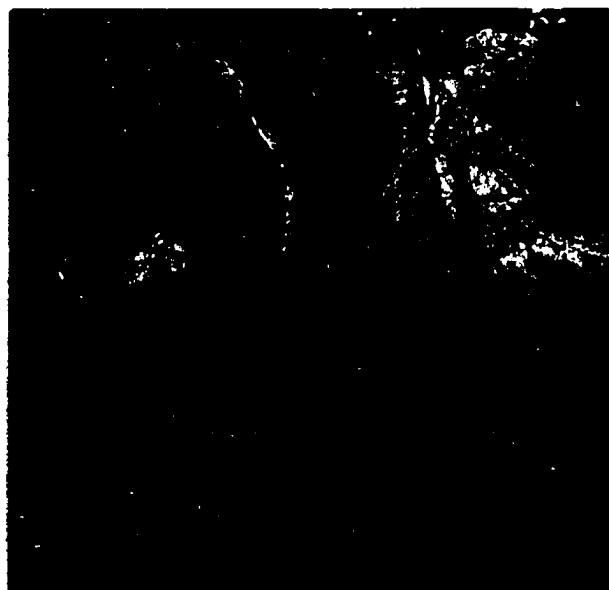


Figure 55. Normal lungs from pig inoculated with 220 m μ filtrate.

Figure 56. Gross pneumonic lesions in right apical and left cardiac lobes of lungs from pig inoculated with 800 m μ filtrate.



controls were tested against virus pneumonia of pigs anti-serum prepared in a boar or in sheep, using the single-diffusion and double-diffusion agar-gel precipitation techniques. No lines of precipitation were observed in the single-diffusion tests observed for a period of 16 days or in the double-diffusion tests observed for 12 days.

Skin sensitivity tests

Eight pigs which had been inoculated with pneumonic lung 6 weeks previously and which were shown to have characteristic gross and microscopic pneumonic lesions at subsequent necropsy, were injected intradermally with 4 antigens prepared by 2 techniques from normal and pneumonic lung. No significant increase in skin thickness was observed during a 72 hour observation period following inoculation. One of the 8 pigs had a temperature of 106.2 F on the second day postinoculation, but 24 hours later the temperature was back to normal. Febrile responses were not observed in the other pigs.

An attempt was made to determine whether a thermal response could be elicited with the antigens prepared from pneumonic lungs. No such reaction was observed in normal or in virus pneumonia infected pigs inoculated intradermally and subcutaneously with the antigens.

Complement fixation test

Sera from sheep which had been inoculated with the agent of virus pneumonia of pigs were tested to determine the presence of complement fixing antibodies to 4 different psittacosis-group antigens. Sera collected prior to inoculation and at 1, 2, 3, 4, and 10 weeks postinoculation were tested. Two sheep remained negative throughout the trial, but in the other 2 low antibody titres were observed against the antigen prepared from the pigeon ornithosis agent grown in duck yolk-sacs. The serum of one sheep which had been immunized by intramuscular injection was positive at a dilution of 1:4 on the 1st, 2nd, 3rd and 4th weeks postinoculation, after which no antibodies could be demonstrated. The serum of one of the sheep immunized by intravenous inoculation also showed a titre of 1:4 on the 1st, 2nd, 3rd and 4th weeks postinoculation after which the serum titre dropped. No antibodies to the other 3 psittacosis group antigens or to the control antigens were demonstrated.

Fluorescent antibody techniques

Using the indirect fluorescent antibody technique, an attempt was made to demonstrate antibodies to the agent of virus pneumonia of pigs in sera from lambs and a boar which had been immunized with suspensions of pneumonic lung. Mycoplasma granularum was included in the trial as a control antigen. Examination of Giemsa stained smears indicated that

very few organisms were present on the slides. No specific fluorescence could be demonstrated after exposure of the antigens to postinoculation sera followed by the appropriate fluorescein-conjugated anti-species sera.

Drug Sensitivity

Tylosin

The prophylactic effect of a single intramuscular injection of Tylosin tartrate in pigs infected with virus pneumonia was determined. At necropsy 4 of the 8 pigs in the untreated virus pneumonia infected group had pneumonic lesions involving approximately from 0.5 to 10 per cent of the lungs. Histological examination revealed lesions typical for virus pneumonia of pigs in all 4 of the pigs with gross lesions, and in 2 of the pigs in which no gross lesions were evident. Six of the 8 Tylosin treated pigs had gross lesions involving 1 to 8 per cent of the lungs, and on microscopic examination were shown to have the characteristic lesions of virus pneumonia of pigs. The gross lesions encountered in both these groups were most frequently present in the cardiac, apical, and intermediate lobes, but they were not confined to these areas.

Three pigs in the uninoculated control group were found to have pneumonic areas involving approximately 1 per cent of the lungs. Microscopic examination revealed that 2 of the cases were typical of purulent bronchopneumonia, but in the

3rd one a few focal accumulations of lymphocytes resembling those seen in virus pneumonia of pigs were observed. A Pseudomonas sp. was recovered from the lungs of this pig. The supply herd was checked for the presence of lesions of virus pneumonia by necropsy of 6 normal pigs of approximately the same age as the experimental group. No lesions were encountered in this group or in any of the uninoculated pigs subsequently necropsied from the herd.

Bacteriological examinations of the lungs were negative for Bordetella bronchiseptica, Mycoplasma spp., Haemophilus spp., Streptococcus spp. and Pasteurella multocida. No cytopathogenic viruses were isolated.

The total feed and water consumption, average daily weight gain and pounds of feed/pounds of gain were calculated for each of the 3 groups of pigs. These figures are presented in Table 2.

Although the average daily weight gain of the infected group is somewhat lower than that of the other groups and the feed conversion efficiency appears to be slightly decreased, the difference is so small that no conclusion can be drawn as to the significance of this finding. A larger number of replicates would be required to determine statistical significance.

The effect of high-level oral administration of Tylosin tartrate on established lesions of virus pneumonia of pigs

Table 1. Lesions in pigs inoculated with filtrates of pneumonic lung suspension

APD of membrane filter	No. of pigs inoculated	No. of pigs with gross lesions	No. of pigs with microscopic lesions
800 m μ	7	7	7
650 m μ	4	4	4
450 m μ	7	5	5
300 m μ	7	5	5
220 m μ	4	0	0

Table 2. Effect of virus pneumonia of pigs infection on feed and water consumption, average daily weight gain and pounds of feed/pounds of gain

Determinations	non-infected group	VPP infected untreated group	VPP infected Tylosin treated group
Total feed consumption, pounds	2850	2850	2750
Total water consumption, gal./pig/day	2.03	2.12	1.93
Average daily weight gain, pounds	1.75	1.66	1.71
Pounds of feed/pounds of gain	2.61	2.74	2.60

was determined. Twelve pigs were divided into 2 groups of 6 pigs on the 21st day after initial exposure, at which time sporadic coughing was observed in both groups. When the pigs were necropsied 7 weeks after treatment, 5 out of 6 untreated pigs had typical gross pneumonic lesions involving approximately 1 to 7 per cent of the lungs. Four out of 6 treated pigs had typical gross lesions involving 1 to 5 per cent of the lungs. The characteristic microscopic lesions of virus pneumonia of pigs were encountered in all of the pigs necropsied. The microscopic lesions encountered in pigs in which no gross lesions were evident are illustrated in Figures 57 and 58.

No Mycoplasma spp., Pasteurella multocida, Haemophilus spp., Streptococcus spp. or Bordetella bronchiseptica were isolated from the lungs.

The pigs which were inoculated with the pooled suspension of lung collected from the Tylosin treated group, were necropsied 8 weeks later. All 3 had gross lesions of virus pneumonia of pigs involving 1 to 7 per cent of the lungs. The typical microscopic lesions of virus pneumonia of pigs were observed in specimens collected from each of the pigs.

Pigs inoculated with the agent of virus pneumonia of pigs while being fed a ration containing Tylosin tartrate were necropsied 21 days after inoculation as was the control group.

Figure 57. Peribronchiolar and perivascular lymphoid hyperplasia and subepithelial infiltration of lymphocytes in bronchiolus. Lesions from pig in which no gross lesions were observed. Hematoxylin and eosin stain. X 100.

Figure 58. Pronounced peribronchiolar and perivascular lymphoid hyperplasia. Alveolar septa appear normal. Lesions from pig in which no gross lesions were observed. Hematoxylin and eosin stain. X 100.



In the untreated control group, 3 of the 4 pigs had characteristic gross lesions of virus pneumonia of pigs involving approximately 1 to 6 per cent of the lungs. The characteristic microscopic lesions of the disease were encountered in all four pigs in the control group. Three of the 4 pigs in the Tylosin treated group had extensive gross lesions characteristic of virus pneumonia of pigs. The typical microscopic lesions of the disease were encountered in the 3 grossly affected lungs. Touch preparations from both groups of pigs contained numerous minute coccoid bodies situated extracellularly in most cases, but occasionally found in the cytoplasm of the cells. These bodies are illustrated in Figures 16 to 21.

An attempt was made to determine the in vitro sensitivity to Tylosin of the organism propagated in artificial medium. No apparent inhibitory effect could be observed even at the highest level of the drug tested, namely 100 µgm./ml.

Furaltadone

The effect of Furaltadone (Valsyn Water Mix) on the agent of virus pneumonia of pigs was determined. The 2 control groups were the same as were used in the first Tylosin trial. In the inoculated, untreated group 6 out of the 8 pigs had microscopic lesions of virus pneumonia of pigs, 4 of them associated with characteristic gross pneumonic lesions. In the uninoculated group, 2 pigs had small foci of purulent

bronchopneumonia, and one had pneumonia resembling virus pneumonia but from which a Pseudomonas sp. was isolated. Characteristic gross lesions of virus pneumonia of pigs were found in the lungs of all the pigs in the group which were started on Valsyn prior to inoculation. These lesions involved from 0.5 to 10 per cent of the lungs. Characteristic microscopic lesions were found in all these pigs. Four of the 9 pigs in the group started on Valsyn 13 days postinoculation had gross lesions involving 0.5 to 8 per cent of the lungs. Microscopic lesions were present in 8 of the 9 pigs in this group.

No Mycoplasma spp., Bordetella bronchiseptica, Haemophilus spp., Streptococcus spp. or Pasteurella multocida were isolated from the lungs of the experimental pigs. No viruses were isolated in cell cultures.

Chlortetracycline

The pigs inoculated with the agent of virus pneumonia of pigs while receiving meal medicated with chlortetracycline were necropsied together with the untreated control group 21 days postinoculation. Three of the untreated control pigs had characteristic gross lesions of virus pneumonia of pigs and the 4th had one small focus which could not with certainty be described as a pneumonic lesion. On histological examination the characteristic lesions were found in all 4 pigs. No gross pneumonic lesions were found in the chlortetracycline treated group, and on microscopic examination, no

changes resembling those associated with virus pneumonia of pigs were observed. Bacteriological examinations were negative, and no coccoid bodies were observed in Giemsa-stained touch preparations from the lungs of the treated group. Several foci containing numerous coccoid bodies were found in the smears prepared from lungs of the untreated control pigs.

Ether Sensitivity

Four pigs inoculated intranasally with an ether-treated suspension of pneumonic lung were necropsied 8 weeks post-inoculation together with 4 control pigs inoculated with untreated lung suspension. Clearly demarcated greyish-pink pneumonic lesions involving approximately 1 to 5 per cent of the lungs were found in each of the control pigs. The lesions occurred primarily in the cardiac and apical lobes, but in one case the intermediate lobe was involved. No gross lesions were encountered in the lungs of pigs inoculated with the ether-treated suspension.

Lesions typical of virus pneumonia of pigs were observed on microscopic examination of lung sections prepared from specimens collected from the control pigs. Very pronounced peribronchial and perivascular lymphoid hyperplasia with very few neutrophiles were observed in 3 out of 4 pigs, but in the 4th one, the neutrophile infiltration was much more pronounced. Occasional giant cells were observed in the pneumonic areas. The microscopic lesions encountered in the

control pigs are illustrated in Figures 59, 60, and 61. The characteristic microscopic lesions of virus pneumonia of pigs were not encountered in the pigs inoculated with the ether-treated suspension. Figure 62 illustrates the microscopic appearance of the lung from a pig inoculated with the ether-treated suspension.

No bacteria were isolated from the specimens collected at necropsy.

Viral Interference

On the 2nd day after inoculation with virus pneumonia of pigs lung suspension mild degenerative changes were observed in the primary swine kidney cell sheets. Distinct cytopathic effects (CPE) were observed on the 2nd day postinoculation in cell sheets inoculated with Newcastle disease virus, Pseudorabies virus, Ohio ECPO-1 virus, and Sturdy virus. Changes were first seen in cells infected with virus BTS-43 on the 3rd day. With all of these viruses the endpoint was reached by the 7th day postinoculation. A very indefinite CPE was observed on the 6th day postinoculation in cell sheets inoculated with Infectious Canine Hepatitis virus. The change was characterized by syncytium formation along the edge of the cell sheets and did not appear to progress rapidly.

Results of the titrations of the 6 viruses are presented in Table 3.

Figure 59. Pronounced peribronchiolar and perivascular lymphoid hyperplasia. Alveolar septa appear normal. Lesions from pig inoculated with pneumonic lung suspension not treated with ether. Hematoxylin and eosin stain. X 100.

Figure 60. Peribronchiolar lymphoid hyperplasia and septal cell proliferation. Lesions from pig inoculated with pneumonic lung suspension not treated with ether. Hematoxylin and eosin stain. X 100.



Figure 61. Perivasicular and peribronchiolar lymphoid hyperplasia, septal cell proliferation and neutrophile infiltration. Lesions from pig inoculated with pneumonic lung suspension not ether-treated. Hematoxylin and eosin stain. X 100.

Figure 62. Lung from pig inoculated with ether-treated pneumonic lung suspension. Note absence of lesions in alveolar septa and in region of bronchioles. Hematoxylin and eosin stain. X 100.



Table 3. Titration of 6 viruses in cells exposed to pneumonia agent 48 hours previously

Viruses	Virus titer ^a	
	Cells exposed to VPP infection	Control cells not exposed to VPP
NDV	4.5	4.3
Pseudorabies	7.0	6.5
Ohio ECPO 1	3.5	4.0
Sturdy	4.0	4.5
BTS-43	2.7	3.0
ICH	1.5	1.0

^aExpressed as Log₁₀ TCID₅₀ per ml.

The final titers indicated that exposure of the cells to the pneumonia agent had not interfered with the growth of any of the 6 viruses tested.

Experimental Mycoplasma pneumoniae
Infection in Swine

Pigs inoculated intranasally with a culture of Mycoplasma pneumoniae showed no febrile response, coughing, or other clinical symptoms during a 6 week period of observation following inoculation. No gross lesions were observed in the lungs, trachea, and nasal turbinates. Small fibrinous peri-toneal adhesions were observed in one pig. Microscopic

examination of sections prepared from lung specimens collected at necropsy revealed no evidence of pneumonia, but only congestion possibly resulting from the electrocution process. Mycoplasma pneumoniae was not reisolated from the lungs, heart surface, peritoneal cavity or nasal turbinates, nor were other bacteria isolated from these specimens.

DISCUSSION

At the initiation of these studies very little information was available on the nature of the etiological agent of virus pneumonia of pigs as it occurs in the U.S.A. Strain No. 11 of the agent was known to be capable of reproducing the characteristic disease. It had been propagated in cell cultures, and had consistently been shown to be free of Bordetella bronchiseptica, Pasteurella multocida, Haemophilus spp., Streptococcus spp., Mycoplasma hyorhinis and swine influenza virus (L'Ecuyer, 1962). It had not been further defined, and it was not known whether this agent was a virus, a psittacosis agent or a Mycoplasma sp.

The viral interference test was performed in an effort to determine whether a competitive relationship existed between the agent and the 6 viruses selected. This was also an attempt to devise a laboratory technique which could be used for titration of the agent. The viruses were selected from several virus groups in an attempt to cover a wide range of virus types, in the hope that interference could be demonstrated. The Newcastle disease virus was the representative of the Myxovirus group selected, the Pseudorabies virus was the Herpesvirus selected, and the Adenovirus group was represented by the Infectious Canine Hepatitis virus. In addition, 3 swine enteroviruses were included in the trial.

No interference could be demonstrated between the agent of virus pneumonia of pigs and the 6 viruses tested. This negative result was not considered as proof that the agent was not related to any of the 6 viruses, but in the light of this finding it was considered unlikely that further studies on viral interference would prove to be profitable.

The results of the ether sensitivity trial demonstrated that the virus pneumonia of pigs agent was sensitive to ethyl ether. Since ether sensitivity is dependent on the presence of lipid-containing surface structures (Andrewes, 1962), this finding suggested that the agent was probably a fairly complex organism possibly possessing a lipid-containing limiting membrane.

The filtration studies which were performed produced interesting results. Five out of 7 pigs in each of the groups receiving the filtrates of the 450 μm and 300 μm APD membranes had characteristic pneumonic lesions. No lesions were observed in the 4 pigs inoculated with the filtrate from the 220 μm APD membrane. While it cannot be stated unequivocally that the smallest forms of the agent did not pass this membrane, it is felt that on the basis of this evidence the conclusion can be drawn that this membrane removed the majority of the infectious particles, while the 300 μm APD membrane passed a sufficiently large number of particles for the filtrate to remain infectious. Estimation of the size of the

agent on the basis of these findings presented some problems. Elford (1933) suggested that since the variables encountered during filtration could not be accurately assessed, a practical procedure was to express the probable limits for the size of the smallest particle just retained by a given membrane under ideal conditions of filtration. For membranes with APD of between 100 $\text{m}\mu$ and 500 $\text{m}\mu$, the size of the retained particles would be 0.5 to 0.75 of the APD of the membrane. If applied to the results of the filtration trial this would indicate an absolute minimum size for the agent of 110 $\text{m}\mu$. Since the largest particle that would not be retained by the 300 $\text{m}\mu$ APD membrane would measure less than 225 $\text{m}\mu$, and since the filtrate of the 300 $\text{m}\mu$ APD membrane was still infective, it can be assumed that the smallest infectious particles measured between 110 and 225 $\text{m}\mu$ in diameter. The fowl-pox virus which was included as a marker virus passed the same membranes as the pneumonia agent. Therefore it must be assumed that there were infectious forms of this virus measuring less than 225 $\text{m}\mu$ in diameter. This figure is slightly less than the 250 $\text{m}\mu$ diameter of fowl-pox virus as determined by electron microscopic techniques (Andrewes, 1964). This is not surprising since size determinations based on electron micrographic measurements tend to be distorted toward increased size.

Three trials were performed to determine the in vivo sensitivity of the virus pneumonia of pigs agent to Tylosin

tartrate. One in vitro trial was performed. In all 4 trials it was shown that this agent was very resistant to the action of the drug. The fact that the organism grows in the presence of 100 $\mu\text{gm.}/\text{ml}$. Tylosin may be useful in developing selective media which can support growth of the agent but not of Mycoplasma hyorhinis and M. granularum, both of which, according to Switzer¹, are sensitive to the drug, M. granularum being very sensitive while M. hyorhinis is somewhat less sensitive.

The results of the chlortetracycline sensitivity trial indicated that the organism was sensitive to this drug, a finding which is in agreement with the reports of several groups of investigators working on swine pneumonia in different parts of the world. The chlortetracycline sensitivity of the agent is indicative of the nonviral nature of the agent of virus pneumonia of pigs. If the chlortetracycline sensitivity of this agent is considered together with the size of the agent as revealed by filtration experiments, it appears that this agent could be either a Mycoplasma sp. or a member of the psittacosis group of agents. In view of this, the Tylosin resistance of the agent is somewhat surprising, since this drug has been shown to be very effective against many members of

¹Dr. W. P. Switzer, Iowa Veterinary Medical Research Institute, Ames, Iowa. Tylosin sensitivity of Mycoplasma spp. Personal communication. 1965.

both the psittacosis group of agents (Pollard and Tanami, 1961) and members of the genus Mycoplasma (Barnes et al., 1960, Yoder et al., 1961, and El Nasri, 1964). It is reasonable to assume, however, that Tylosin resistant agents may exist in both these groups.

An attempt was made to demonstrate antibodies to agents of the psittacosis group in sera from lambs inoculated with the agent of virus pneumonia of pigs. Two of the 4 sheep remained negative throughout the trial, but the other two developed low antibody titers to the pigeon ornithosis agent between the 1st and 4th weeks postinoculation. Since no anamnestic response was observed following booster inoculations, the antibody response was considered to be nonspecific.

Failure to demonstrate antibodies to the agent of virus pneumonia of pigs in sera from sheep or swine inoculated with the agent indicates that the antigen used in the immunization procedure was of low antigenicity. Both the agar-gel diffusion micro-precipitation test and the fluorescent antibody technique are highly sensitive serological systems, and even low-grade antibody responses should have been detected using these systems. The lung tissue in the crude pneumonic lung antigen probably masked the infectious agent, thus preventing effective stimulation of antibody production. The isolation of the infectious agent in cell-free media should allow preparation of better antigens and antisera, and the development of

specific serological procedures for diagnostic and experimental use.

Failure to demonstrate a specific skin reaction in virus pneumonia infected pigs inoculated intradermally with antigens prepared from pneumonic lung can probably also be attributed to the low antigenicity of the antigens. The antigen preparation techniques were selected since they represented proven methods of skin-test antigen preparation for a Mycoplasma sp. and for the psittacosis agents. The febrile response observed in one pig could not be repeated, and was considered to be nonspecific.

The results of the filtration experiments revealed that the smallest infective form of the pneumonia agent measured between 110 and 225 $\text{m}\mu$ in diameter. While an agent measuring 225 $\text{m}\mu$ would be just within the limits of resolution of the conventional light microscope and would therefore be difficult to visualize, it was considered reasonable to assume that larger forms of the agent existed, and if this were so, they should be visible in pneumonic lesions and in cell cultures inoculated with the agent. Examination of Giemsa-stained touch preparations from pneumonic lesions revealed numerous small bluish-purple coccoid to cocco-bacillary organisms and occasional ring forms. The organisms were frequently difficult to find, but when a focus of organisms was found, numerous colonies were usually encountered in the immediate area.

The reason for the focal distribution in touch preparations became evident when on examination of Giemsa stained sections of pneumonic lung it was demonstrated that the organisms were most numerous on the surface of the bronchiolar epithelium, and were infrequently found in the alveoli. The foci of organisms probably represented those areas of the touch preparations which were derived from the bronchioli. Intracellular accumulations of the organisms were not frequent and were probably the result of phagocytosis and not representative of a developmental phase of the organism. Since the organisms were usually encountered extracellularly, it was assumed that they were not obligatory intracellular parasites. The size, morphology and extracellular occurrence of the organisms was considered to be strong evidence in favor of their inclusion in the genus Mycoplasma.

Failure to demonstrate the organisms in smears prepared from older lesions is interpreted as indicating a very low-grade infection rather than the absence of the infectious agent.

The organisms encountered in the touch preparations resemble in size and morphology the pleomorphic organisms or "P.O.'s" observed by Goodwin and Whittlestone (1963a) in cell cultures infected with a causative agent of virus pneumonia of pigs.

Cell cultures which were established from pneumonic

lesions were stained and examined microscopically for microorganisms. Occasional extracellular and intracellular clumps of the organisms were seen, but in view of the fact that the cell cultures were subsequently shown to be infective by pig inoculation, the numbers of organisms were surprisingly low. This probably resulted from the washing off of extracellular organisms during the staining process.

The acridine orange staining technique which was used in an attempt to determine the nucleic acid composition of the pneumonia agent produced rather indefinite results. Focal intracytoplasmic accumulations of reddish-orange staining material believed to be RNA were encountered in infected tissues. This change was not consistently encountered in infected tissues and was never seen in normal cells. The changes observed were interpreted as representative of a cellular reaction to the causative agent and not as part of the cyclic development of the organism. The significance of the clumps of green-staining material observed in some cells in infected tissues is not known, but it is believed that this material may represent phagocytosed DNA-containing remnants of nuclei of degenerated cells.

Electron microscopic examination of pneumonic lung revealed a marked increase in the number of free-lying and attached septal cells. The electron-dense, membrane-bound bodies which were occasionally encountered in these cells or

lying free in the alveolar spaces were believed to be the infectious agent. The possibility that these bodies represent a change produced by the agent rather than the agent itself can not be eliminated. In size and morphology these bodies closely resembled the elementary bodies of the trachoma agent as described by Mitsui et al. (1964), and the meningopneumonitis agent described by Erlandson and Allen (1964). Both of these agents are members of the psittacosis group. However, the intracellular developmental stages characteristically associated with the psittacosis group of agents were not seen in the virus pneumonia of pigs preparations.

A morphological resemblance also existed between the bodies observed in virus pneumonia of pigs and the "elementary bodies" of Mycoplasma spp. as described by Domermuth et al. (1964a, 1964b) and the intracellular Mycoplasma described by Dmochowski et al. (1964). The intracytoplasmic vesicular bodies which were occasionally observed in virus pneumonia of pigs closely resembled the structures described by Dmochowski et al. (1964) as "Mycoplasma containing primary bodies."

The amorphous material present in the alveolar spaces of pneumonic lung probably represented an abnormal accumulation of the lipo-protein surfactant material (Bensch et al., 1964) secreted by the septal cells. The abnormal amount of this material could be the result of increased production due to the large number of septal cells, or to interference in the

normal outflow of this substance resulting from blockage of the pores of Kohn.

The intracytoplasmic bodies encountered on microscopic examination of infected cell cultures also resembled both the Mycoplasma spp. and the agents of the psittacosis group. The membranous whorls which were present in the organisms resembled similar structures observed in elementary and initial bodies of the meningopneumonitis agent by Erlandson and Allen (1964). These authors commented on the similarity between these structures and the mesosomes of bacteria, but did not present proof of their involvement in the process of intracellular respiration. The significance of the whorls in these objects is not known.

Microscopic examination of hematoxylin-eosin stained sections from experimentally produced lesions of virus pneumonia of pigs consistently revealed peribronchiolar and perivascular lymphoid hyperplasia. The peribronchiolar lesions were consistently more prominent than the perivascular ones, and the blood-vessels associated with the lymphoid hyperplasia were usually those situated in close proximity to the bronchioles. Thus the lymphoid hyperplasia, which is considered characteristic of this disease, may be the result of penetration of the bronchiolar epithelium by toxins produced by the organisms. This would explain the predominant peribronchiolar localization of the cellular response. Actual penetration of the lung parenchyma by the organisms may play only a minor role in the

pathogenesis of the lesions.

Lesions in which pronounced alveolar interstitial thickening, giant cell formation, neutrophile infiltration, septal cell proliferation, as well as the peribronchiolar and perivascular lymphoid hyperplasia were observed, were considered to represent the active phase of the infection. These changes were prominent in pneumonic lesions in pigs necropsied 3 to 4 weeks postinoculation. The lesions characterized by pronounced lymphoid hyperplasia associated with very few other changes were believed to represent a more chronic phase of the infectious process. This type of lesion was commonly encountered in pigs necropsied 4 to 8 weeks postinoculation.

The apparent increase in virulence of the agent which was observed after serial passage in pigs is difficult to explain. It is reasonable to assume that passage of the organism as it occurs in nature, involves infection of swine with varying degrees of resistance. The organism would thus come into contact with a number of body defense mechanisms, some of them possibly capable of modifying or attenuating the strain. Furthermore, the actual dosage of the agent received by a pig during the natural transmission process is probably relatively low, thus allowing the body defenses to either overcome it or to limit its pathogenic effect.

The relatively rapid experimental passage of the organism which was accomplished by intranasal inoculation of highly

susceptible pigs with large doses of the infectious agent could have resulted in an actual increase in virulence. An alternative explanation for the apparent increase in virulence would be that as a result of serial passage in highly susceptible pigs, using large dosages of the agent, an increase in the titer of the organism had occurred. The dosage of the organisms in suspensions of pneumonic lung would therefore have become increasingly larger, resulting in greater infectivity.

The reasons for the frequent occurrence of giant cells in lesions after rapid serial passage in pigs are not known. This phenomenon could be attributed to the presence of high toxin concentrations resulting from increased virulence of the infectious agent or to an increase in the number of organisms in the inoculum. Betts et al. (1955) described a pneumonia of swine in which large numbers of giant cells were encountered. The disease differed clinically from virus pneumonia of pigs and the microscopic lesions were also considered to be different. Hjärre et al. (1952a) occasionally encountered giant cells in lesions of swine influenza and "enzootic virus pneumonia." A giant cell pneumonia which was accompanied by vegetative dermatitis was also described by Hjärre et al. (1952b). Although the lesions which they described were consistent with those of virus pneumonia of pigs, they did not imply that the two diseases were in any way similar.

Giant cells have thus not been consistently associated with the lesions of the disease in the past. On the basis of the findings presented here it is suggested that perhaps they are representative of an acute phase of the uncomplicated disease, a state which is rarely encountered in the field.

The causative agent of virus pneumonia of pigs was successfully propagated in cell cultures. While the cell cultures established from pneumonic lung were still infective after 3 serial passages, no pneumonic lesions were elicited by 7th or 8th serial passage material. These results confirm the findings of L'Ecuyer and Switzer (1953) who were not able to propagate this strain of the agent beyond the 4th serial passage in HeLa cells. These findings indicated that the growth environment was definitely sub-optimal. Since the agent has a predilection for lung tissue it was postulated that an increased oxygen environment might favor growth of the organism, but no enhancement of growth was observed in cell cultures grown in an atmosphere consisting of 95 per cent oxygen and 5 per cent carbon dioxide. It is possible that the oxygen level used was too high.

Microscopic examination of stained coverslips of cell cultures established from pneumonic lungs revealed relatively few of the cocco-bacillary organisms. This finding possibly indicates that growth was primarily occurring extracellularly.

Repeated attempts were made to propagate the infectious

agent in embryonated hens eggs, all without success. This finding confirms the results obtained by L'Ecuyer (1962). Similarly, no definite evidence of infectivity of this agent for mice could be demonstrated. The mortality which was observed in one group of inoculated baby mice could not be reproduced and was considered to be the result of an intercurrent infection.

A small coccobacillary organism isolated in cell-free media from pneumonic lung was found to elicit the characteristic gross and microscopic lesions associated with virus pneumonia of pigs, and has been demonstrated to be the causative agent present in strain No. 11 of this disease. Lesions were reproduced with the agent after 8 serial passages which constituted a dilution of 10^{-9} of the original lung suspension. Daily examination of culture tubes revealed a definite increase in the numbers of organisms present. When these observations are considered together with the fact that the serial passages constituted a total exposure of the organism to 37 C for 25 days, there is no doubt that growth and multiplication of the organism actually occurred.

The name Mycoplasma hyopneumoniae (new species) is proposed for the organism. Its inclusion in the genus Mycoplasma is based on the characteristic colony morphology observed, and on the size, morphology, staining characteristics and penicillin resistance of the microorganism. Failure to

revert to a bacterial form when propagated continuously in the absence of bacterial inhibitors further favors its inclusion in the genus Mycoplasma. The species name hyopneumoniae indicates that it is the Mycoplasma of swine pneumonia.

None of the results of the earlier experiments conflict with the concept of the pneumonia agent being a Mycoplasma sp. The organisms visualized by light and electron microscopy in known infected tissues resembled Mycoplasma spp. in size and morphology. The results of size determinations by filtration indicated that the agent was either a Mycoplasma sp., a psittacosis agent or a large virus. The predominant extracellular occurrence of the organisms as seen in stained touch preparations and sections of lesions favored its inclusion in the genus Mycoplasma. The ether sensitivity of the agent is believed to be compatible with its designation as a Mycoplasma sp. since these organisms are very delicate membrane-bound structures which would most probably be sensitive to ether. However, no reference to ether sensitivity determinations on Mycoplasma spp. was encountered in the literature perused. The chlortetracycline sensitivity of the agent was also consistent with its inclusion in the genus Mycoplasma.

The relationship between M. pneumoniae and M. hyopneumoniae (n. sp.) has not been determined. An unsuccessful attempt was made to elicit pneumonic lesions in respiratory disease-free pigs by intranasal instillation of M. pneumoniae.

The negative results do not preclude a close relationship between the two organisms.

Several attempts were made to propagate the pneumonia agent in media designed to support growth of other swine and avian Mycoplasma spp. and M. pneumoniae. The fact that these attempts were repeatedly unsuccessful indicates the extreme fastidiousness of the agent and suggests that it is distinct from M. hyorhinis, M. granularum and M. pneumoniae. It also differs from the other swine Mycoplasma spp. in its Tylosin resistance.

Bakos and Dinter (1963) isolated an agent in cell-free media from swine enzootic pneumonia in Sweden. They considered this agent to be a Mycoplasma sp., but did not present evidence of its ability to elicit the characteristic disease on sub-inoculation. Goodwin and Whittlestone (1964c) in England produced enzootic pneumonia in pigs with an agent grown in cell-free media. They did not identify the agent but suggested that it might be a Mycoplasma sp.

When the results presented in this thesis are considered in conjunction with the findings of the above-mentioned authors incriminating nonviral causative agents for the disease widely known as virus pneumonia of pigs, the legitimate use of "virus" in the nomenclature of the disease becomes questionable. It is suggested that if further investigations reveal widespread occurrence of M. hyopneumoniae (n. sp.) as

the causative agent of this disease, a more specific name such as "Pulmonary Mycoplasmosis of Swine" or "Mycoplasmal Pneumonia of Swine" should be adopted.

The isolation in cell-free media of the pneumonia agent and its identification as a Mycoplasma sp. was achieved after the major portion of the work presented in this thesis had been completed. If this had been accomplished earlier a more refined antigen would have been available for the characterization studies. However, the fact that the agent can now be grown in cell-free media should allow rapid advances in research on this very important disease.

There are several important problems which have to be overcome at the outset. In the first place it is essential that better growth media be developed since more abundant growth of the organism would make available antigens suitable for preparation of antisera. These antisera would prove valuable in the development of serological procedures for diagnostic purposes as well as for laboratory use.

More abundant growth of the agent would also allow the assay of drugs for possible use in prophylaxis or therapy of the disease. Methods of attenuation or inactivation of the agent for use in prophylactic immunization could be developed, since recent work with M. pneumoniae has indicated that a satisfactory vaccine may be developed for this agent.

The development of improved diagnostic procedures for use in the identification of M. hyopneumoniae should receive high priority, since this will facilitate field surveys to determine the extent of the etiological role of this organism in chronic pneumonia of swine.

SUMMARY

An infectious agent capable of eliciting the disease known as virus pneumonia of pigs (VPP) was isolated, grown in cell-free media and characterized. The agent was isolated from characteristic pneumonic lesions in a pig from a farm with a typical field outbreak of the disease. The agent was consistently shown to be free of bacteria and viruses cytopathogenic for swine kidney and swine lung cell cultures.

An unsuccessful attempt was made to demonstrate interference between the pneumonia agent and Newcastle Disease virus, Pseudorabies virus, Infectious Canine Hepatitis virus, and 3 swine enteroviruses.

The infectious agent was shown to be sensitive to ethyl ether, a fact which was interpreted as being indicative of the presence of lipid-containing surface structures.

Filtration studies which were performed to determine the size of the infectious agent revealed that the agent would pass a 300 μ APD membrane but not one with 220 μ APD. It was estimated that the smallest infectious particle measured between 110 μ and 225 μ in diameter.

The in vivo sensitivity of the agent to Tylosin tartrate, Furaltadone and chlortetracycline was determined. The agent was sensitive to chlortetracycline, but no sensitivity to the other 2 drugs could be demonstrated. No in vitro sensitivity to Tylosin tartrate was observed.

An unsuccessful attempt was made to demonstrate a serological relationship between the pneumonia organism and the psittacosis agents.

Both the fluorescent antibody technique and the agar-gel diffusion technique were used in unsuccessful attempts to demonstrate antibodies to the pneumonia agent in sera from sheep and a pig which had been repeatedly inoculated with the agent. No skin hypersensitivity reaction could be demonstrated in infected pigs injected intradermally with antigens prepared from pneumonic lung.

Small coccoid to cocco-bacillary organisms and occasional ring forms were observed in Giemsa stained touch preparations and sections from pneumonic lung, as well as in cell cultures infected with the agent.

Electron-microscopic examination of infected tissues revealed round to oval electron-dense bodies which were believed to represent the infectious agent. The morphological similarities between these structures, Mycoplasma spp. and psittacosis agents was discussed.

The pneumonia agent was propagated in cell cultures, but serial passage beyond the 3rd passage level was not achieved.

The infectious agent was successfully propagated in cell-free fluid medium and on solid media. Surface growth was markedly enhanced by the presence of a Micrococcus sp. nurse colony. The characteristic colony morphology associated with

the Mycoplasma spp. was observed when the agent was grown on solid media.

After 7 and also 8 serial passages in cell-free media the characteristic gross and microscopic lesions of virus pneumonia were elicited in respiratory disease-free pigs inoculated with the organism.

The name Mycoplasma hyopneumoniae (new species) was proposed for the organism. Its inclusion in the genus Mycoplasma was based on the characteristic colony morphology, and on the size, morphology, staining characteristics and penicillin resistance of the organism. The species name hyopneumoniae indicates that it is the Mycoplasma of swine pneumonia.

It was suggested that if further investigations reveal wide-spread involvement of Mycoplasma hyopneumoniae as the causative agent of virus pneumonia of pigs, a more specific name such as "Pulmonary Mycoplasmosis of Swine" or "Mycoplasma Pneumonia of Swine" should be adopted.

LITERATURE CITED

- Andrewes, C. H. 1962. Classification of viruses of vertebrates. *Advances in Virus Research* 9:271-296.
- Andrewes, C. H. 1964. Viruses of vertebrates. London, England. Bailliere, Tindall and Cox.
- Andrewes, C. H. and Horstmann, D. M. 1949. The susceptibility of viruses to ethyl ether. *Journal of General Microbiology* 3:290-297.
- Aristotle. 329 B.C. *Historia animalium*. In R. M. Hutchins, ed. Great books of the Western World. Vol. 2. pp. 129-130. Chicago, Illinois, Encyclopedia Britannica, Inc.
- Armstrong, J. A. and Reed, S. E. 1964. Nature and origin of initial bodies in lymphogranuloma venereum. *Nature* 201: 371-373.
- Armstrong, J. A., Valentine, R. C., and Fildes, C. 1963. Structure and replication of the trachoma agent in cell cultures as shown by electron microscopy. *Journal of General Microbiology* 30:59-73.
- Bakos, K. and Dinter, Z. 1963. Das SEP agens. *World Veterinary Congress*, 17th, Hannover, Proceedings. 1:549-554.
- Bakos, K., Obel, A., Swahn, O., and Walzl, H. 1960. "Inclusion-body-rhinitis" bei Ferkeln mit experimenteller Enzootischer Pneumonie. *Zentralblatt für Veterinärmedizin* 7:262-272.
- Barnes, L. E., Ose, E. E., and Gossett, F. O. 1960. Treatment of experimental PPLO infections in young chickens with tyllosin, a new antibiotic. *Poultry Science* 39:1376-1381.
- Barwell, C. F. 1952. Some observations on the antigenic structure of psittacosis and lymphogranuloma venereum viruses. *British Journal of Experimental Pathology* 33: 258-279.
- Bedson, S. P. and Bland, J. O. W. 1932. A morphological study of psittacosis virus, with the description of a developmental cycle. *British Journal of Experimental Pathology* 13:461-466.

- Bensch, K., Schaefer, K., and Avery, M. E. 1964. Granular pneumocytes: electron microscopic evidence of their exocrinic function. *Science* 145:1318-1319.
- Betts, A. O. 1952. Respiratory diseases of pigs. V. Some clinical and epidemiological aspects of virus pneumonia of pigs. *Veterinary Record* 64:283-288.
- Betts, A. O. 1953. Pneumonic diseases of the pig caused by viruses: a review of the literature. *British Veterinary Journal* 109:99-106.
- Betts, A. O. and Beveridge, W. I. B. 1952. Investigation on a virus pneumonia of long duration prevalent in pigs. *Journal of Pathology and Bacteriology* 64:247-248.
- Betts, A. O. and Campbell, R. C. 1956. The action of antibiotics and sulphamethazine on the causal agent of virus pneumonia of pigs. *Journal of Comparative Pathology and Therapeutics* 66:89-101.
- Betts, A. O. and Whittlestone, P. 1963. Enzootic or virus pneumonia of pigs: the production of pneumonia with tissue culture fluids. *Research in Veterinary Science* 4:471-479.
- Betts, A. O., Whittlestone, P., and Beveridge, W. I. B. 1955. Investigations on the control of virus pneumonia of pigs (VPP) in the field. *Veterinary Record* 67:685-688.
- Bontscheff, N. 1961a. Atiologische Forschungen der Virus-Pneumonie bei den Ferkeln. *Zentralblatt für Bakteriologie* 181:417-422.
- Bontscheff, N. 1961b. Kultivierung des Ferkelpneumonie-Virus in Huhnerembryonen und Erforschung seiner Eigenschaften. *Zentralblatt für Bakteriologie* 181:423-428.
- Börnfors, S. and Lannek, N. 1955. Therapeutic trials in enzootic pneumonia in pigs. *Nordisk Veterinaermedicin* 7:15-31.
- Börnfors, S. and Lannek, N. 1958. Studies on the immunity to swine enzootic pneumonia. *Nordisk Veterinaermedicin* 10:426-430.
- Carter, G. R. and Schroder, J. D. 1956. Virus pneumonia of pigs in Canada, with special reference to the role of pleuropneumonialike organisms. *Cornell Veterinarian* 46:344-354.

- Caulfield, J. B. 1957. Effects of varying the vehicle for OsO_4 in tissue fixation. *Journal of Biophysical and Biochemical Cytology* 3:827-829.
- Chalquest, R. R. 1962. Cultivation of the infectious-synovitis-type pleuropneumonia-like organisms. *Avian Diseases* 6:36-43.
- Chanock, R. M., Hayflick, L., and Barile, M. F. 1962a. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. *National Academy of Sciences Proceedings* 48:41-49.
- Chanock, R. M., James, W. D., Fox, H. H., Turner, H. C., Mufson, M. A., and Hayflick, L. 1962b. Growth of Eaton PPLO in broth and preparation of complement fixing antigen. *Society for Experimental Biology and Medicine Proceedings* 110:884-889.
- Clyde, W. A. 1961. Demonstration of Eaton's agent in tissue culture. *Society for Experimental Biology and Medicine Proceedings* 107:715-718.
- Clyde, W. A. 1963. Hemolysis in identifying Eaton's pleuropneumonia-like organism. *Science* 139:55.
- Dinter, Z., Bakos, K., and Björklund, N. E. 1954. Über das Verhalten des Virus der enzootischen Pneumonie der Schweine in der Lunge dieser Tierart. *Monatshefte für Tierheilkunde* 6:21-30.
- Dinter, Z., Wesslén, T., and Iannek, N. 1957. Die Kultivierung des SEP-agens im Dottersack des Bruteis. *Zentralblatt für Veterinärmedizin* 4:581-587.
- Dmochowski, L., Grey, C. E., Dreyer, D. A., Sykes, J. A., Langford, P. L., and Taylor, H. G. 1964. Mycoplasma (pleuropneumonia-like organisms) and human leukemia. *Medical Records and Annals* 57:563-568.
- Domermuth, C. H., Nielsen, M. H., Freundt, E. A., and Birch-Andersen, A. 1964a. Ultrastructure of Mycoplasma species. *Journal of Bacteriology* 88:727-744.
- Domermuth, C. H., Nielsen, M. H., Freundt, E. A., and Birch-Andersen, A. 1964b. Gross morphology and ultrastructure of *Mycoplasma gallisepticum*. *Journal of Bacteriology* 88:1428-1432.

- Dowdle, W. R. and Robinson, R. Q. 1964. An indirect hemagglutination test for diagnosis of Mycoplasma pneumoniae infections. Society for Experimental Biology and Medicine Proceedings 116:947-950.
- Dulbecco, R. and Vogt, M. 1954. Plaque formation and isolation of pure lines of poliomyelitis virus. Journal of Experimental Medicine 99:167-182.
- Duncan, J. R. 1965. The pathogenesis of Bordetella bronchiseptica rhinitis and pneumonia in swine. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.
- Earle, W. R., Schilling, E. L., Stark, T. H., Straus, N. P., Brown, M. F., and Shelton, E. 1943. Production of malignancy in vitro. IV. The mouse fibroblast cultures and changes seen in the living cells. United States National Cancer Institute Journal 4:165-212.
- Eaton, M. D. 1950. Action of aureomycin and chloromycetin on the virus of primary atypical pneumonia. Society for Experimental Biology and Medicine Proceedings 73:24-29.
- Eaton, M. D., Meiklejohn, G., and van Herick, W. 1944. Studies on the etiology of primary atypical pneumonia. I. Filterable agent transmissible to cotton rats, hamsters and chick embryos. Journal of Experimental Medicine 79:649-668.
- Eaton, M. D., Meiklejohn, G., van Herick, W., and Corey, M. 1945. Studies on the etiology of primary atypical pneumonia. II. Properties of the virus isolated and propagated in chick embryos. Journal of Experimental Medicine 82:317-328.
- Edwards, G. A. and Fogh, J. 1960. Fine structure of pleuropneumonia-like organisms in pure culture and in infected tissue culture cells. Journal of Bacteriology 79: 267-276.
- El Nasri, M. 1964. The effect of tylosin against experimental and natural infection with Mycoplasma in goats. Veterinary Record 76:876-877.
- Elford, W. J. 1933. The principles of ultrafiltration as applied in biological studies. Royal Society of London Proceedings, Series B, 112:384-406.

- Erlandson, R. A. and Allen, E. G. 1964. The ultrastructure of meningopneumonitis. *Virology* 22:410-418.
- Fogh, J. and Fogh, H. 1964. A method for direct demonstration of pleuropneumonia-like organisms in cultured cells. *Society for Experimental Biology and Medicine Proceedings* 117:899-901.
- Fontaine, M., Goret, P., Brion, A., Pilet, Ch., Girard, M., and Alloui, P. 1959. Nouvelles recherches expérimentales sur la pneumonie à virus du porc. *Académie Vétérinaire de France Bulletin* 32:675-694.
- Freundt, E. A. 1960. Morphology and classification of the PPLo. *New York Academy of Sciences Annals* 79:312-325.
- Fulton, J. S., Burton, A. N., and Millar, J. L. 1953. Virus pneumonia in swine. *American Veterinary Medical Association Journal* 123:221-224.
- Gaylord, W. H. 1954. Intracellular forms of meningopneumonitis virus. *Journal of Experimental Medicine* 100: 575-580.
- Goodwin, R. F. W. and Whittlestone, P. 1960. Experiences with a scheme for supervising pig herds believed to be free from enzootic pneumonia (virus pneumonia). *Veterinary Record* 73:1029-1054.
- Goodwin, R. F. W. and Whittlestone, P. 1962. A respiratory disease of pigs (type XI) differing from enzootic pneumonia: the field disease. *Journal of Comparative Pathology and Therapeutics* 72:389-410.
- Goodwin, R. F. W. and Whittlestone, P. 1963a. Production of enzootic pneumonia in pigs with an agent grown in tissue culture from the natural disease. *British Journal of Experimental Pathology* 44:291-299.
- Goodwin, R. F. W. and Whittlestone, P. 1963b. The differential diagnosis of respiratory diseases in the pig. *World Veterinary Congress, 17th, Hannover, Proceedings* 1:549-554.
- Goodwin, R. F. W. and Whittlestone, P. 1964a. A respiratory disease of pigs (type XI) differing from enzootic pneumonia: natural transmission experiments. *Journal of Comparative Pathology and Therapeutics* 74:8-16.

- Goodwin, R. F. W. and Whittlestone, P. 1964b. A respiratory disease of pigs (type XI) differing from enzootic pneumonia: artificial transmission experiments. *Journal of Comparative Pathology and Therapeutics* 74:308-319.
- Goodwin, R. F. W. and Whittlestone, P. 1964c. Production of enzootic pneumonia in pigs with a micro-organism grown in media free from living cells. *Veterinary Record* 76:611-613.
- Goodwin, R. F. W. and Whittlestone, P. 1965. Experiments on the possible relationship between inclusion-body rhinitis, enzootic pneumonia and type-XI pneumonia of pigs. *Research in Veterinary Science* 6:100-107.
- Goret, P., Fontaine, M., Brion, A., Pilet, C., Girard, M. and Legrand, P. 1960. Recherches expérimentales sur la prévention et le traitement de la pneumonie à virus du porc par le chlorhydrate de tetracycline. *Recueil de Médecine Vétérinaire* 136:711-730.
- Gourlay, R. N. 1964. The allergic reaction in contagious bovine pleuropneumonia. *Journal of Comparative Pathology and Therapeutics* 74:286-299.
- Gulrajani, T. S. and Beveridge, W. I. B. 1951. Studies on respiratory diseases of pigs. IV. Transmission of infectious pneumonia and its differentiation from swine influenza. *Journal of Comparative Pathology and Therapeutics* 61:118-139.
- Hancock, B. B., Bohl, E. H., and Birkeland, J. M. 1959. Swine kidney cell cultures: susceptibility to viruses and use in isolation of enteric viruses of swine. *American Journal of Veterinary Research* 20:127-132.
- Hänichen, T. 1964. Untersuchungen über die Veränderungen am Bronchial- und Alveolarepithel bei der enzootischen Virus-pneumonie des Schweines: zugleich ein Beitrag zur Genes primärer Lungengeschwülste. *Zentralblatt für Veterinärmedizin* 11:251-269.
- Hanks, J. H. 1948. The longevity of chick tissue cultures without renewal of medium. *Journal of Cellular and Comparative Physiology* 31:235-260.

- Hilleman, M. R., Hamparian, V. V., Ketler, A., Reilly, C., McClellan, L., Cornfeld, D., and Stokes, J. 1962. Acute respiratory illness among children and adults: field study of contemporary importance of several viruses and appraisal of the literature. American Medical Association Journal 180:445-453.
- Hjärre, A. 1957. Über die enzootische Viruspneumonie beim Schwein. Monatshefte für Veterinärmedizin 12:503-508.
- Hjärre, A., Dinter, Z., and Bakos, R. 1951. Influensa och influensaliknonde sjukdomar hos svin. Nordiska Veterinärmötet 6:7-27.
- Hjärre, A., Dinter, Z., and Bakos, K. 1952a. Vergleichende Untersuchungen über eine influenza-ähnliche Schweinkrankheit in Schweden und Shopes Schweineinfluenza. Nordisk Veterinaermedicin 4:1025-1045.
- Hjärre, A., Dinter, Z., and Bakos, K. 1954. Über Zuchtungsversuche mit dem Schweineinfluenzavirus (Shope) und dem Virus der enzootischen Schweinepneumonie in vitro. Nordisk Veterinaermedicin 6:919-935.
- Hjärre, A., Ehlers, T., and Thal, E. 1952b. Riesenzellennepmonien bei Tieren. Schweizerische Zeitschrift für Allgemeine Pathologie und Bakteriologie 15:566-590.
- Howatson, A. F. and Almeida, J. D. 1958. A method for the study of cultured cells by their sectioning and electron microscopy. Journal of Biophysical and Biochemical Cytology 4:115-117.
- Howell, D. G. and Gordon, W. S. 1954. Cold agglutinins in the serum of pigs. Veterinary Record 66:318.
- Kajima, M., Sharon, N., and Pollard, M. 1964. Electron microscopy of latent psittacosis virus in McCoy cells. Journal of Bacteriology 88:709-715.
- Kellenberger, E., Ryter, A., and Sechaud, J. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. Journal of Biophysical and Biochemical Cytology 4: 671-678.

- Kerr, K. M., Mascoli, C. C., Olson, N. O., and Campbell, A. 1964. Rapid specific agglutination of Eaton agent (Mycoplasma pneumoniae). Journal of Bacteriology 87: 478-479.
- Konno, S., Okaniwa, A., and Fujiwara, H. 1963. Occurrence of swine enzootic pneumonia in Japan. National Institute of Animal Health Quarterly 3:217-223.
- Kushida, H. 1961. A new embedding method for ultrathin sectioning using a methacrylate resin with three dimensional polymer structure. Journal of Electronmicroscopy 10: 194-197.
- Lannek, N. and Börnfors, S. 1956. Prophylactic treatment of enzootic pneumonia (virus pneumonia) in pigs with tetracycline and oxytetracycline. Veterinary Record 68:53-55.
- Lannek, N. and Börnfors, S. 1957. Immunity to enzootic pneumonia in pigs following recovery from the disease. Nordisk Veterinaermedicin 9:91-98.
- Lannek, N. and Wesslén, T. 1955. Histological examination of tissue cultures inoculated with a cytopathogenic agent from swine enzootic pneumonia. Acta Pathologica et Microbiologica Scandinavica 36:343-351.
- Lannek, N. and Wesslén, T. 1957. Evidence that the SEP agent is an etiological factor in enzootic pneumonia in swine. Nordisk Veterinaermedicin 9:177-190.
- L'Ecuyer, C. L. 1962. Propagation of the primary agent of virus pneumonia of pigs and determination of the microbiology of naturally occurring cases. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.
- L'Ecuyer, C. L. and Switzer, W. P. 1963. Virus pneumonia of pigs; attempts at propagation of the causative agent in cell cultures and chicken embryos. Canadian Journal of Comparative Medicine and Veterinary Science 27:91-99.
- L'Ecuyer, C. L., Switzer, W. P., and Roberts, E. D. 1961. Microbiological survey of pneumonic and normal swine lungs. American Journal of Veterinary Research 22: 1020-1025.
- Lewis, P. A. and Shope, R. E. 1931. Swine influenza. II. A hemophilic bacillus from the respiratory tract of infected swine. Journal of Experimental Medicine 54:361-373.

- Liu, C. 1957. Studies on primary atypical pneumonia. I. Localization, isolation and cultivation of a virus in chick embryos. Journal of Experimental Medicine 106: 455-467.
- Luft, J. H. 1961. Improvements in epoxy resin embedding methods. Journal of Biophysical and Biochemical Cytology 9:409-414.
- Mackenzie, A. 1963. Experimental observations on lungworm infection together with virus pneumonia in pigs. Veterinary Record 75:114-116.
- Madin, S. H., Andriese, P. C., and Darby, N. B. 1957. The in vitro cultivation of tissues of domestic and laboratory animals. American Journal of Veterinary Research 18: 932-941.
- Marcus, P. E., Cieciura, S. J., and Puck, T. T. 1956. Clonal growth in vitro of epithelial cells from normal tissues. Journal of Experimental Medicine 104:615-628.
- Marmion, B. P. and Goodburn, G. M. 1961. Effect of an organic gold salt on Eaton's primary atypical pneumonia agent and other observations. Nature 189:247-248.
- Meiklejohn, G. 1943. The cold agglutination test in the diagnosis of primary atypical pneumonia. Society for Experimental Biology and Medicine Proceedings 54:181-184.
- Melnick, J. L. 1955. Tissue culture techniques and their application to original isolation, growth, and assay of poliomyelitis and orphan viruses. New York Academy of Sciences Annals 61:754-773.
- Mitsui, Y., Fujimoto, M., and Kajima, M. 1964. Development and morphology of trachoma agent in the yolk sac cell as revealed by electron microscopy. Virology 23:30-45.
- Mitsui, Y., Kajima, M., Nishimura, A., and Konishi, K. 1962. Morphology of trachoma agent in conjunctiva and chick embryo. New York Academy of Sciences Annals 98:131-144.
- Monberg-Jørgensen, H. C. 1938. Die Aetiologie der Ferkelgrippe. Infektionsversuche mit bakterienfreien Gradocolmembranfiltraten. Berliner und Münchener Tierärztliche Wochenschrift, Jahrgang 1938:573-576.

- Morowitz, H. J., Tourtellotte, M. E., Guild, W. R., Castro, E., and Woese, C. 1962. The chemical composition and sub-microscopic morphology of Mycoplasma gallisepticum, avian PPL. Journal of Molecular Biology 4:93-103.
- Nelson, S. L. 1961. Pneumonia of swine. Microfilm Copy No. 61-5749, Unpublished Ph.D. thesis, Purdue University, Lafayette, Indiana. Ann Arbor, Michigan, University Microfilms.
- Obel, A. L. 1961. Über Lungenvänderungen bei "Inclusion-Body Rhinitis" des Schweines. Zentralblatt für Veterinärmedizin 8:509-522.
- Omar, A. R. 1964. The characteristic cells of the lung and their reaction to injury. Veterinary Bulletin 34: 371-382; 431-443.
- Ouchterlony, O. 1949. Antigen-antibody reactions in gels. Acta Pathologica et Microbiologica Scandinavica 26: 507-515.
- Ouidin, J. 1948. L'analyse immunochimique qualitative: Méthodes par diffusion des antigènes au sein de l'immunsérum précipitant gélosé. Institute Pasteur Annales (Paris) 75:30-51.
- Page, L. A. and Bankowski, R. A. 1960. Factors affecting the production and detection of ornithosis antibodies in infected turkeys. American Journal of Veterinary Research 21:971-978.
- Page, L. A., Storz, J., and Pangborn, J. 1961. Electron microscopic survey of thirteen strains of Miyagawanella. Avian Diseases 5:121-131.
- Palade, G. E. 1952. A study of fixation for electron microscopy. Journal of Experimental Medicine 95:285-298.
- Palyusik, M. 1953. Untersuchungen über die Ätiologie der Enzootischen Ferkelpneumonie. Acta Veterinaria (Hungarica) 3:63-73.
- Pattison, I. H. 1956. A histological study of a transmissible pneumonia of pigs characterised by extensive lymphoid hyperplasia. Veterinary Record 68:490-494.
- Penny, H. C. 1954. The treatment of virus pneumonia of the pig with chloramphenicol. Veterinary Record 66:730-731.

- Penttinen, K. and Rislakki, V. 1953. Om den i Finland förekommande smittosamma grishostans etiologi. II. Adaptsionsförsök i embryonerade hönsögg och serologiska undersökningar. Nordisk Veterinaermedicin 5:125-130.
- Plowright, W. 1953. Observations on virus pneumonia of pigs in Kenya. Veterinary Record 65:313-318.
- Pollard, M. and Tanami, Y. 1961. Effects of antibiotics on trachoma virus in tissue cultures. Society for Experimental Biology and Medicine Proceedings 107:508-511.
- Pollard, M. and Tanami, Y. 1962. Cytochemistry of trachoma virus replication in tissue cultures. New York Academy of Sciences Annals 98:50-61.
- Pugh, R. J. 1956. Chloramphenicol in virus pleuropneumonia of pigs. Veterinary Record 68:1048-1049.
- Pullar, E. M. 1948. Infectious pneumonia of pigs. I. General description, differential diagnosis, and epidemiology. Australian Veterinary Journal 24:320-330.
- Pullar, E. M. 1949a. Infectious pneumonia of pigs. II. Morbidity, incidence, type, and location of lesions. Australian Veterinary Journal 25:53-60.
- Pullar, E. M. 1949b. Infectious pneumonia of pigs. III. Transmission experiments and a field trial of a formalin killed vaccine. Australian Veterinary Journal 25:123-130.
- Reagan, R. L., Brueckner, A. L., and Delaplane, J.-P. 1951. Morphological observations by electron microscopy of the virus (Tucker strain) of a chronic respiratory disease in chickens. Poultry Science 30:113-115.
- Reagan, R. L., Day, W. E., and Brueckner, A. L. 1953. Electron microscopy studies of four strains of chronic respiratory agent. Poultry Science 32:960-965.
- Reed, L. J. and Muench, H. 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene 71:250-257.
- Reimann, H. A. 1964. Viral and mycoplasmal pneumonias: prevention and treatment. Diseases of the Chest 46: 158-164.

- Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology 17:208-212.
- Rislakki, V. 1953. Om der i Finland förekommande smittosamma grishostans eitologi. I. Infektionsförsök med försöksgrisar. Nordisk Veterinaermedicin 5:113-124.
- Roberts, E. D., Switzer, W. P., and L'Ecuyer, C. L. 1962. Influence of Pasteurella multocida and Mycoplasma hyorhinis (PPLO) on the histopathology of field cases of swine pneumonia. Cornell Veterinarian 52:306-327.
- Ross, R. F. 1965. Incidence of Bordetella bronchiseptica in swine and experimental production of rhinitis with the organism. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology.
- Ross, R. F. and Switzer, W. P. 1963. Comparison of isolates of Mycoplasma hyorhinis by indirect hemagglutination. American Journal of Veterinary Research 24:622-627.
- Roth, L. E. 1961. A method for reducing methacrylate sublimation from thin sections. Journal of Ultrastructural Research 5:142-150.
- Ruys, A. C. and van Iterson, W. 1961. Some characteristics of pathogenic avian PPLO. Antonie van Leeuwenhoek Journal of Microbiology and Serology 27:129-138.
- Sabatini, D. D., Bensch, K., and Barnett, R. J. 1963. Cytochemistry and electron microscopy; the preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. Journal of Cell Biology 17:19-58.
- Segre, D. 1964. Cytopathic effects of normally non-cytopathogenic viruses in tissue cultures held under increased oxygen pressure. Society for Experimental Biology and Medicine Proceedings 117:567-569.
- Shifrine, M., Pangborn, J., and Adler, H. E. 1962. Colonial growth of Mycoplasma gallisepticum observed with the electron microscope. Journal of Bacteriology 83:187-192.
- Shope, R. E. 1931a. Swine influenza. I. Experimental transmission and etiology. Journal of Experimental Medicine 54:349-359.
- Shope, R. E. 1931b. Swine influenza. III. Filtration experiments and etiology. Journal of Experimental Medicine 54:373-385.

- Somerson, N. L., Taylor-Robinson, D., and Chanock, R. M. 1963. Hemolysin production as an aid in the identification and quantitation of Eaton agent (Mycoplasma pneumoniae). American Journal of Hygiene 77:122-128.
- Surdan, C. and Sorodoc, G. 1960. Studies on the virus of the enzootic virus pneumonia of the swine. Acta Microbiologica Academiae Scientiarum Hungaricae 7:187.
- Switzer, W. P. 1955. Studies on infectious atrophic rhinitis. IV. Characterization of a pleuropneumonia-like organism isolated from the nasal cavities of swine. American Journal of Veterinary Research 16:540-544.
- Switzer, W. P. 1956. Studies on infectious atrophic rhinitis. V. Concept that several agents may cause turbinate atrophy. American Journal of Veterinary Research 17: 478-484.
- Switzer, W. P. 1959. Action of certain viruses, Mycoplasma hyorhinis, and nasal trichomonads on swine tissue cultures. American Journal of Veterinary Research 20: 1010-1019.
- Switzer, W. P. and L'Ecuyer, C. L. 1960. Detection of swine nasal viruses in cell cultures. American Journal of Veterinary Research 21:967-970.
- Tajima, M., Nomura, Y., and Kubota, Y. 1957. Structure and development of viruses of the psittacosis-lymphogranuloma group observed in the electron microscope. Journal of Bacteriology 74:605-620.
- Tanami, Y., Pollard, M., and Starr, J. J. 1961. Replication pattern of psittacosis virus in a tissue culture system. Virology 15:22-29.
- Thomas, L., Mirick, G. S., Curnen, E. C., Ziegler, J. E., and Horsfall, F. L. 1943. Serological reactions with an indifferent streptococcus in primary atypical pneumonia. Science 98:566-568.
- Trautwein, G. 1960. Histopathologie und Histochemie der chronischen Viruspneumonie des Schweines. Deutsche Tierärztliche Wochenschrift 67:435-441.
- Underdahl, N. R. and Kelley, G. W. 1957. The enhancement of virus pneumonia of pigs by the migration of Ascaris suum larvae. American Veterinary Medical Association Journal 130:173-176.

- Urman, H. K., Underdahl, N. R., and Young, G. A. 1958. Comparative histopathology of experimental swine influenza and virus pneumonia of pigs in disease-free, antibody-devoid pigs. American Journal of Veterinary Research 19:913-917.
- U. S. Armed Forces Institute of Pathology. 1960. Manual of histologic and special staining technics. 2nd ed. New York, McGraw-Hill Book Company Inc.
- Van Iterson, W. and Ruys, A. C. 1960. On the nature of PPLO. II. Electron microscopy. Antonie van Leeuwenhoek Journal of Microbiology and Serology 26:9-22.
- Watson, M. L. 1958. Staining of tissue sections for electron microscopy with heavy metals. Journal of Biophysical and Biochemical Cytology 4:475-478.
- Wesslén, T. and Lannek, N. 1954. The isolation and cultivation in tissue culture of a cytopathogenic agent from pigs with enzootic pneumonia (socalled virus pneumonia). Nordisk Veterinaermedicin 6:481-499.
- Whittlestone, P. 1957. Some respiratory diseases of pigs. Veterinary Record 69:1354-1363.
- Yoder, H. W. and Hofstad, M. S. 1964. Characterization of avian mycoplasma. Avian Diseases 8:481-512.
- Yoder, H. W., Nelson, C. L., and Hofstad, M. S. 1961. Tylosin, an effective antibiotic for treatment of PPLO turkey sinusitis. Veterinary Medicine 56:178-180.
- Young, G. A. 1964a. Virus pneumonia of pigs. In H. W. Dunne, ed. Diseases of swine. 2nd ed. pp. 127-135. Ames, Iowa, Iowa State University Press.
- Young, G. A. 1964b. Viral infections of domestic animals. Annual Review of Microbiology 18:269-300.
- Young, G. A. and Underdahl, N. R. 1960. Certification of swine herds as virus pneumonia-free. American Veterinary Medical Association Journal 137:186-189.

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